Biochemical characterization of a-amino acid ester hydrolases

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The cover picture is a photomicrograph of amoxicillin crystals, one of the antibiotics the α-amino acid ester hydrolases can make. The picture is used with permission of M.W. Davidson, National High Magnetic Field Laboratory, Florida State University.

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For all those times you stood by me
For all the truth you made me see
For all the joy you brought to my life
…..
(Diane Warren)

Ter nagedachtenis aan mijn vader

Contents

1

Introduction

The relation of β-lactam antibiotics, β-lactam acylases and α-amino acid ester hydrolases

BIOCATALYSIS

The remarkable catalytic power of enzymes, which combines a broad chemical scope, a high selectivity, a high rate enhancement and a high efficiency, makes them attractive catalysts for numerous conversions in industrial production of food, feed or fine chemicals.

For centuries enzymes have been used in the form of fermentative organisms for the production of wine, cheese or bread. This is reflected in the name "enzyme", which was introduced by F. W. Kuhne in 1878 to address the catalytic forces that were proposed to be present in living organisms and is derived from the Greek phrase 'en zymē' meaning "in leaven" (Bohinski, 1987; Stryer, 1995). Despite the world-wide integration of enzymes in the manufacturing of food, it was not until the late nineteenth and early twentieth century that the mystery of enzymes was little by little elucidated and that their important biological role and industrial usefulness became evident. It started with the first reports of catalytic forces or enzymatic reactions outside living cells. In 1894, Fisher described the selective conversion of α-methylglucoside to glucose with a filtered aqueous extract of yeast (Fisher, 1894). Additionally, the Buchner brothers observed rather by surprise the conversion of sucrose into alcohol by cell-free extracts of yeast in 1897 (Buchner, 1897). More information about the nature of enzymes was obtained in 1926, when Sumner gained an enzyme (urease) for the first time in its pure crystalline form. His claim that an enzyme was a protein substance was received with a lot of scepticism at that time, but the isolation of more enzymes by Sumner and other researchers led to the world-wide acceptance of the protein nature of enzymes in 1935 (Bohinski, 1987). How enzymes worked remained unclear for many additional years although Fisher envisioned that enzymes and substrates fitted like a lock and a key, which proved to be a rather accurate metaphor to illustrate the specificity of an enzyme. More insight in the binding of substrates and enzymatic mechanism was obtained when the first three-dimensional structure of an enzyme (lysozyme) was solved in 1965. In the mean time theories to describe the enzyme kinetics evolved and resulted in the famous and still used equations proposed by Michaelis and Menten in 1913. Today, more than 2500 enzymes have been identified and characterized of which around 250 are used as biocatalysts in a commercial process either as pure enzymes or present in intact microbial cells (Woodley, 2000).

The first industrial-valuable single-enzymatic conversion is considered the hydroxylation of steroids which was performed with cells of *Rhizopus nigricans* and was introduced in 1952 (Woodley, 2000). However, ideally pure enzymes were employed. Unfortunately, enzymes were known as very sensitive and vulnerable to degradation and inactivation, which hampered their introduction on a large industrial scale for many years. A turning point is considered the creation of insoluble enzymes by immobilizing them on inert supports, which started in the 1960s. The immobilization technique facilitated easy recovery and reuse of enzymes and increased their stability (Woodley, 2000). Additionally, when Klibanov discovered in 1978 that enzymes can also work in organic solutions (Laere, 1996), the idea of enzymes being unworkable was weakened. In the mean time the growing awareness of the environment and the associated governmental regulations forced industry to implement cleaner or so-called greener

technology. Since the use of enzymes, either in isolated form or as whole-cell preparations, enables very efficient transformations in an aqueous environment (no-organic solvents needed) with few by-products, a rapid increase of the number of industrially implemented biocatalytic processes in the 1980s followed. At present, technological development in molecular biology, protein engineering and high-throughput screening allow the development of stable biocatalysts with tailor-made activity and selectivity, which surely will result in more industrial applications in the future (Schmid *et al*., 2001; Zaks, 2001).

Nowadays, enzymes are applied in the manufacturing of food, animal feed, detergents, pulp and paper, clothing and fine chemicals. An illustration of the application of an enzyme in the clothing industry is the replacement of washing with pumic stones by treatment with cellulase to give denim clothes their popular aged appearance. Cellulase removes the blue dye indigo attached to the surface of the fabric in a much more gentle way, which is beneficial for the strength of the garments and the industrial washing machines. An example of a compound that is produced with the assistance of an enzyme in the food industry can be found in the production of the artificial sweetener aspartame, in which the remarkable specificity of the enzyme thermolysin is effectively used. Thermolysin selectively couples L-phenylalanine methyl ester from a racemic mixture to the α-carboxyl group of an N-protected aspartic acid. Subsequent removal of the protective group of the produced dipeptide results in aspartame (Schmid *et al*., 2001). The selectively of thermolysin is essential as coupling of D-phenylalanine methyl ester would lead to a product with a bitter taste.

A key application of immobilised biocatalysts can be found in the fine chemical industry in the cleavage by penicillin acylases of penicillin G (or V) to yield 6-aminopenicillanic acid (6-APA). This compound is needed for the production of semi-synthetic penicillins (Woodley, 2000), which still is predominantly produced chemically. However, also in this process the implementation of enzymes is ongoing (Bruggink, 2001; Bruggink *et al*., 1998). For example, the subject of our thesis, α-amino acid ester hydrolases (AEHs), are able to synthesise semi-synthetic antibiotics and are potentially very interesting to use as a biocatalyst. Before we will discuss these biocatalytically-interesting enzymes and the synthesis processes in more detail, an introduction to β-lactam antibiotics will be given.

β-LACTAM ANTIBIOTICS

Discovery of β-lactam antibiotics

Several scientists observed the inhibition on bacterial growth by contaminating molds, before Alexander Fleming discovered a fungus able to inhibit the growth of *Staphylococcus aureus* in 1928. He studied the fungus in more detail, identified it as *Penicillium notatum*, and called the anti-bacterial substance penicillin. Although Fleming used the penicillin-containing broth filtrate to show that the substance was not toxic to rabbits and could cure an eye infection, he mainly saw penicillin as a natural antiseptic (Bennet and Chung, 2001; Diggins, 2000). It was not until 1939 that the scientists Howard W. Florey and Ernst B. Chain purified penicillin and undoubtedly demonstrated its therapeutic value (Abraham, 1981; Bennet and Chung, 2001). World War II stimulated further research and in

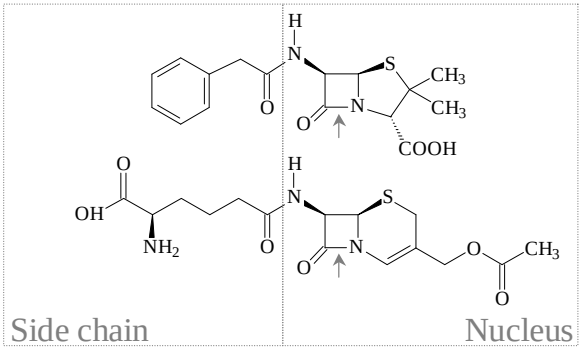


Figure 1. Structure of the β-lactam antibiotics penicillin G (1) and cephalosporin C (2). The acyl moiety is referred to as the side chain (left box); the ring structures form the so-called nucleus (right box). The arrow indicated the pharmacologically essential β-lactam bond. The border between the side-chain and nucleus box indicates the bond cleaved by β-lactam acylases.

1942 the first large-scale fermentation of a *Penicillium* mold was performed in the United States. The subsequent availability of larger quantities of penicillin allowed effective treatment of infected wounds, which saved many soldiers from death during the war.

The industrially introduced production-strain *Penicillium chrysogenum* could produce next to penicillin G (Fig. 1, (**1**)) five other β-lactam antibiotics, which differed only in their side chain (structures not shown). Research showed that the yield of a specific antibiotic could be increased by the addition of a precursor of its side chain, such as phenyl acetic acid in case of penicillin G. A search for possible side chain precursors led to the biosynthetic and acid stable penicillin V, which was obtained by adding phenoxy acetic acid to the fermentation broth (Savidge, 1984). Currently, about 16,000 tons of fermentative penicillin G and V are produced annually for therapeutic purposes (Bruggink, 2001).

A new penicillin-like antibiotic was discovered in 1948, when Giuseppe Brotzu isolated the mould *Cephalosporium acremonium* from a sewer pipe in Sardinia. Crude filtrates of this organism displayed the same activity against *S. aureus* as shown for the *Penicillium* strains and the antibacterial substance was named cephalosporin C (Nicholas *et al.*, 1995). The relation between these antibiotics became evident from their chemical structures (Fig. 1), which showed the conservation of a common β-lactam ring. In penicillin G (**1**) the β-lactam ring is connected to a thiazolidine ring and in cephalosporin C (**2**) to a six-membered ring completing the so-called β-lactam nucleus. To the β-lactam nulei so-called side chains are attached, a phenylacetyl group in penicillin G and a D-2-aminoadipyl moiety in cephalosporin C. The side chain strongly influences the antibacterial spectrum and the pharmacological properties of a β-lactam antibiotic. Depending on the nucleus, the β-lactam antibiotic is either referred to as a penicillin or a cephalosporin. Nowadays, the β-lactam antibiotics are the most prescribed and effective drugs for bacterial infections and for their discovery of the almost non-toxic penicillin G, Fleming, Florey and Chain shared the Nobel Prize for physiology and medicine in 1944.

Mode of action

The β-lactam antibiotics are such effective antibacterial agents, mainly because they interfere with the synthesis of the bacterial cell wall. Depending on the type of bacterium, the cell wall consists of one or two lipid bilayers surrounded by an insoluble and strong peptidoglycan layer (Fig. 2) (Schlegel, 1995). The peptidoglycan layer consists of peptidoglycan chains, which are formed by the repeating amino sugars N-acetylglucosamine (NAG) and N-acetylmuramic

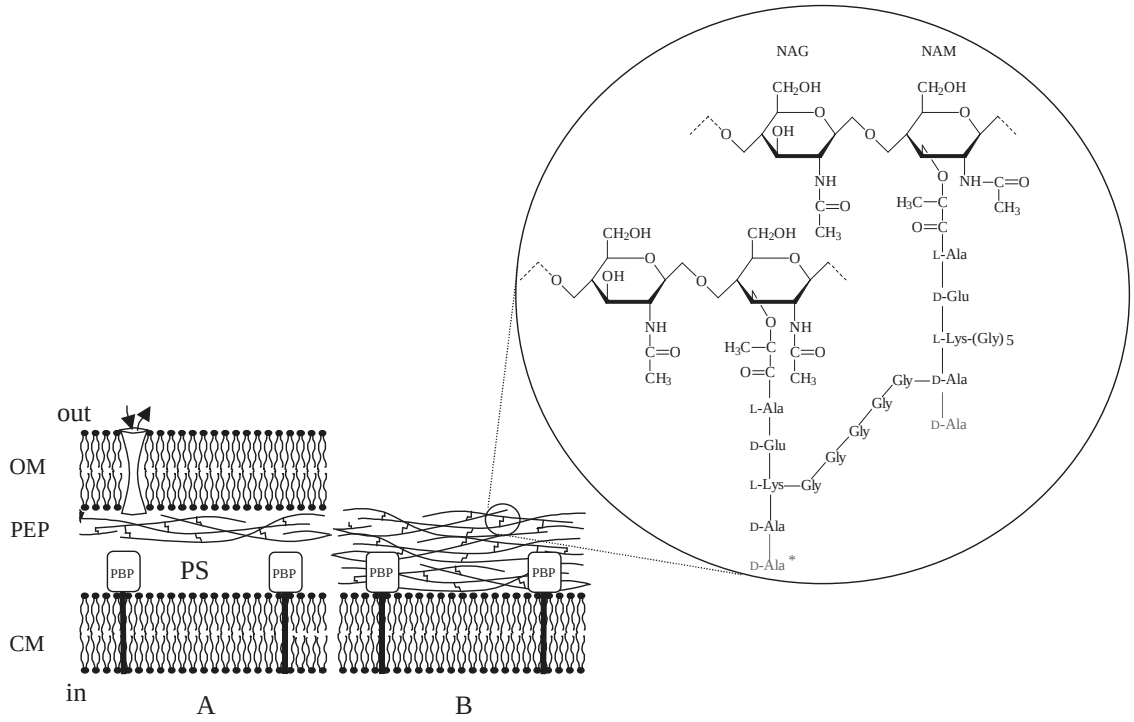


Figure 2. Schematic representations of the bacterial gram-negative (A) and gram-positive (B) cell wall. The outer membrane (OM) and cytoplasmic membrane (CM) both consist of a lipid bilayer with membrane proteins, some of which are forming pores in the membrane through which substances can diffuse in and out of the cell (freely adapted from [Nicholas, 1995 #3]). In the circle a detailed representation of the peptidoglycan cross link in *S. aureus* is given (freely adapted from [Bohinski, 1987 #128]). Other abbreviations: PEP, peptidoglycan layer; PS, periplasmic space; PBP, penicillin-binding proteins; NAG, N-acetyl glucosamine and NAM, N-acetyl muramic acid. The D-Ala residue in grey is cleaved off by transpeptidase, the grey D-Ala with the asterisk is cleaved of by carboxypeptidase.

acid (NAM) with a variable peptide chain attached to the carboxyl group of the NAM-unit (Fig. 2, inset). The peptidoglycan chains are covalently cross-linked to each other via short peptide-bridges that stretch out from the third position of the peptide chain and is connected to the carboxyl group of a D-alanine residue from another peptide chain (Fig. 2, inset). This cross-linking is catalysed by transpeptidases, which react with the D-alanyl-D-alanine (D-Ala-D-Ala) moiety of the peptide attached to the NAM-unit to form a covalent acyl-enzyme complex upon release of the terminal D-alanine (Fig. 3, reaction 1). Subsequent aminolysis of this complex by the terminal amino group of the bridging peptide chain of another peptidoglycan results in the cross-link. To complete the cross-link, the terminal D-Ala group in the D-Ala-D-Ala moiety of the main peptide of the other peptidoglycan is removed by carboxypeptidases (Fig 2, inset) via hydrolysis of a similar covalent acyl enzyme complex. The β-lactam antibiotics interfere with this process, as they are substrate analogues for these enzymes. They mimic the D-Ala-D-Ala moiety in which the amide bond in the β-lactam ring resembles the peptide bond of this dipeptide (Fig. 2). The peptidases are tempted by the highly reactive character of the β-lactam bond and react with the antibiotic, forming a covalent intermediate (Fig. 3,

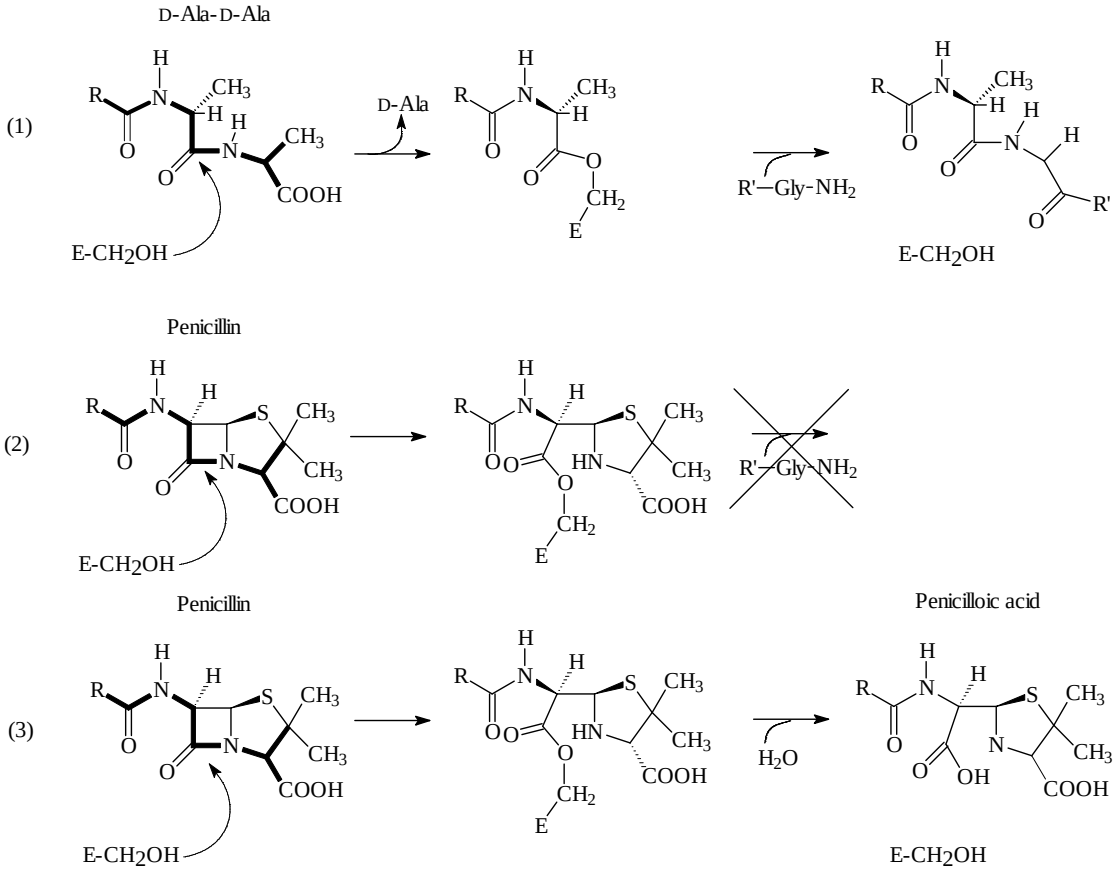


Figure 3. Reaction scheme of the enzyme (E) transpeptidase with its substrate the D-Ala-D-Ala terminus of a peptidoglycan unit (1) and its inhibitor penicillin (2). The inactivation of penicillin to penicilloic acid by β-lactamase (E in 3) is also shown, R: remainder of a peptide. The bold line illustrates the high degree of resemblance between the natural substrate (1) and the inhibitor penicillin.

reaction 2). However, due to its high stability this penicilloyl-enzyme intermediate does not react further, irreversibly inactivating the enzyme. In this way β-lactam antibiotics covalently bind to a set of different enzymes, the so-called penicillin binding proteins (PBPs). Bacteria contain many different PBPs, each playing an important role in cell morphology and/or viability. By inactivating the transpeptidases and carboxypeptidases, a peptidoglycan layer with insufficient cross-links is formed, which is not strong enough to withstand the high internal osmotic pressure. Bacterial autolysins degrade these peptidoglycan strands, a process that proceeds unchecked in the presence of β-lactam antibiotics and eventually results in lysis of the growing bacterial cells that are exposed to the antibiotics (Abraham, 1981; Nicholas *et al.*, 1995).

Resistance to β-lactam antibiotics

The first bacteria resistant to penicillin G were reported soon after the introduction of this antibiotic in medical practice (Abraham, 1981). Research showed that these bacteria thank their resistance mainly to the expression of the enzyme penicillinase (β-lactamase) that inactivates the penicillins by hydrolysing the β-lactam bond (Fig.

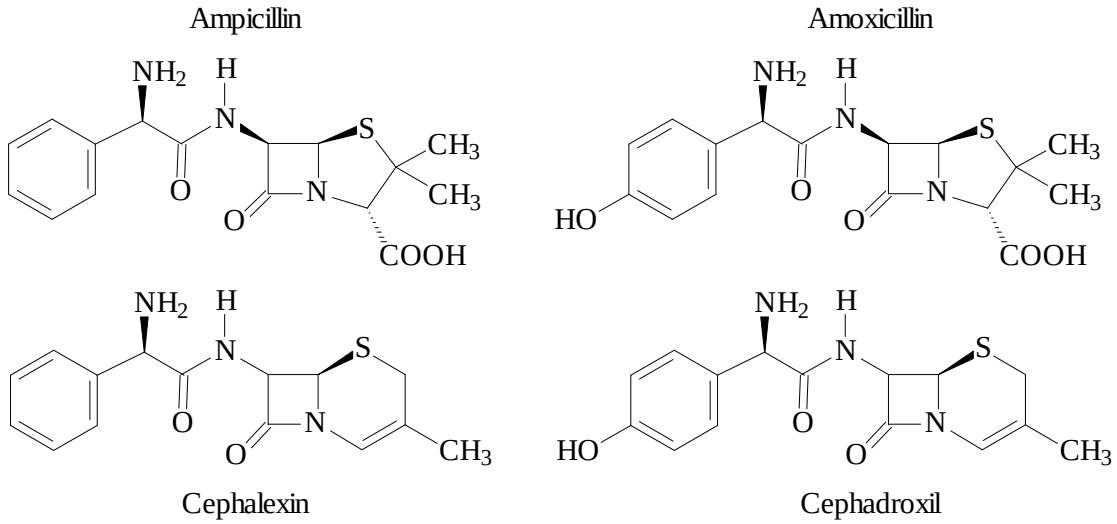


Figure 4. Examples of semi-synthetic β–lactam antibiotics. Shown are penicillins (upper) and cephalosporins (lower) with phenylglycine derived side chains.

3, reaction 3). In doing so, the β-lactamase forms a covalent intermediate with penicillin in the same way as the peptidases. However, in contrast to the penicilloyl-complexes of the PBPs, the intermediate with a β-lactamase is easily hydrolysed, resulting in inactive penicilloic acid and the recycled enzyme. Up to now more than 250 β-lactamases have been identified, which are either plasmid or chromosomally encoded and vary in their substrate range, inhibition profiles, enzymatic properties and molecular structure. Especially, the plasmid-encoded β-lactamases contribute to bacterial resistance spreading as the plasmids can easily be transferred to other Gram negative or Gram positive bacteria by conjugation or by the action of transducing phages, respectively (Essack, 2001; Samaha-Kfoury and Araj, 2003). It is generally accepted that due to the intensive use of penicillin G in the 1950s, the β-lactamase genes spread rapidly under pressure of natural selection. Additionally, the β-lactamases may rapidly adapt via point mutations to obtain activity with newly introduced β-lactam compounds (Samaha-Kfoury and Araj, 2003). As a result, the expression of β-lactamases is the most widespread and the most efficient bacterial mechanism to withstand the lethal action of β-lactam antibiotics.

Another defence mechanism which bacteria often employ to fight β-lactam antibiotics is changing the affinity of the essential PBPs for β-lactam antibiotics by mutation or recombination (Essack, 2001; Nicholas *et al.*, 1995). Alternatively, gram-negative bacteria can alter the proteins that form the channels in their outer membrane in such a way that the diffusion of the β-lactam antibiotics through the membrane to reach the PBPs located in the perpiplasmic space is hindered, increasing the resistance significantly (Nicholas *et al.*, 1995).

Semi-synthetic β-lactam antibiotics

In response to the evolution of β-lactamases and to meet the demand for antibiotics that have a broader antibacterial spectrum and/or improved pharmacological properties, the search

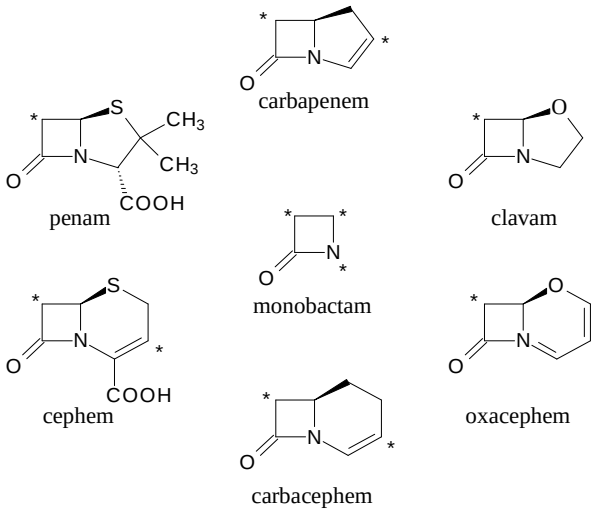


Figure 5. The basic β-lactam nuclei. The asterisks indicate positions with variable substituents among the individual antibiotics.

for new β-lactam antibiotics was started soon after the world-wide introduction of penicillin G. The elucidation of the structure of biosynthetic β-lactam antibiotics with a broader antibacterial spectrum (penicillin N (a D-α-amino adipyl side chain connected to a 6-APA nucleus)) or with a higher acid stability (penicillin V (a phenoxymethyl side chain and a 6-APA nucleus)), indicated that variations in the side chain can alter the properties of a β-lactam antibiotic (Abraham, 1981; Vandamme and Voets, 1974). A breakthrough for the synthesis of new β-lactam antibiotics was the production of the free β-lactam nucleus by a *Penicillium* strain grown in the absence of side chain precursors in 1959 (Bruggink, 2001; Vandamme and Voets, 1974). Coupling of a synthetic 2,6-dimethyoxybenzoyl side-chain to the free 6-APA nucleus resulted in the first semi-synthetic β-lactam antibiotic methicillin (1960) that was effective against penicillin G resistant bacteria. Other clinically valuable semi-synthetic β-lactam antibiotics were obtained by the coupling of, for example, phenylglycine and hydroxyphenylglycine resulting in ampicillin (1961, Fig. 4), which has a broader activity spectrum against gram-negative bacteria, and amoxicillin (1972), an antibiotic with a high oral absorption, respectively (Bruggink, 2001; Nicholas *et al.*, 1995). Cephalosporin C, with a 7-aminocephalosporanic acid nucleus (7-ACA, Fig.1 (**2**)), was a less effective antibiotic but appeared resistant to inactivation by β-lactamase activity (Abraham, 1987). Variations in the 3-acetoxy substituents of the cephalosporin nucleus in combination with the different side chains allowed for a much wider search for effective antibiotics and resulted in four generations of cephalosporins with improved and or specific properties. The explored combinations of side chains and nuclei have resulted in more than 50 penicillins and up to 70 clinically useful cephalosporins (Neu, 1992). Next to the penicillin (penam) and cephalosporin (cephem) nuclei, several other nuclei (Fig. 5) from novel β-lactam antibiotics have been identified increasing the opportunities in the continuous fight against bacterial pathogens. Nowadays, the allergic reactions induced in some individuals are considered the major drawback of β-lactam antibiotics. However, the numerous possible combinations of synthetic side chains and β-lactam nuclei allow for large varieties in both the bacterial effectiveness and pharmacological properties.

β-LACTAM ACYLASES

The first enzyme able to cleave the amide bond in β-lactam antibiotics between the β-lactam nucleus and a carboxylic acid functionality, leaving the cyclic β-lactam amide bond intact was

described as early as 1950 (penicillin G acylase from *Escherichia coli* ATCC 11105). Ten years later, the ability of the same enzyme to catalyse the reverse reaction (condensation) allowing formation of (semi-synthetic) β-lactam antibiotics was described (Vandamme and Voets, 1974). From then on, more enzymes displaying this activity with similar or different substrate ranges have been found in several prokaryotic species.

The classical nomenclature of these so-called β-lactam acylases is inspired by industrially relevant properties and based on the preferred β-lactam antibiotic in hydrolysis. First, they are divided in penicillin and cephalosporin acylases, according to the nucleus of their preferred substrate, 6-aminopenicillanic acid (6-APA), or 7-aminocephalosporanic acid (7-ACA) andderivatives thereof, respectively. Further grouping into subclasses is then based on the preferred side chain (Fig. 6). Since the affinity of the β-lactam acylases for the nucleus is very low, an alternative nomenclature based on the preferred acyl moiety has been proposed, naming them α-acylamino-β-lactam acylhydrolases classes I to IV (Fig. 6) (Nam *et al.*, 1985). The substrate-specificity approach in both these nomenclatures is considered out dated as now more knowledge about the amino acid sequences, the molecular structure and catalytic mechanism of β-lactam acylases is available. However, in the literature still the classical nomenclature is used for the β-lactam acylases and according to that we will describe these enzymes in more detail.

Penicillin acylases

The penicillin acylases are, according to their side chain preference, further divided in three classes, penicillin G, penicillin V and ampicillin

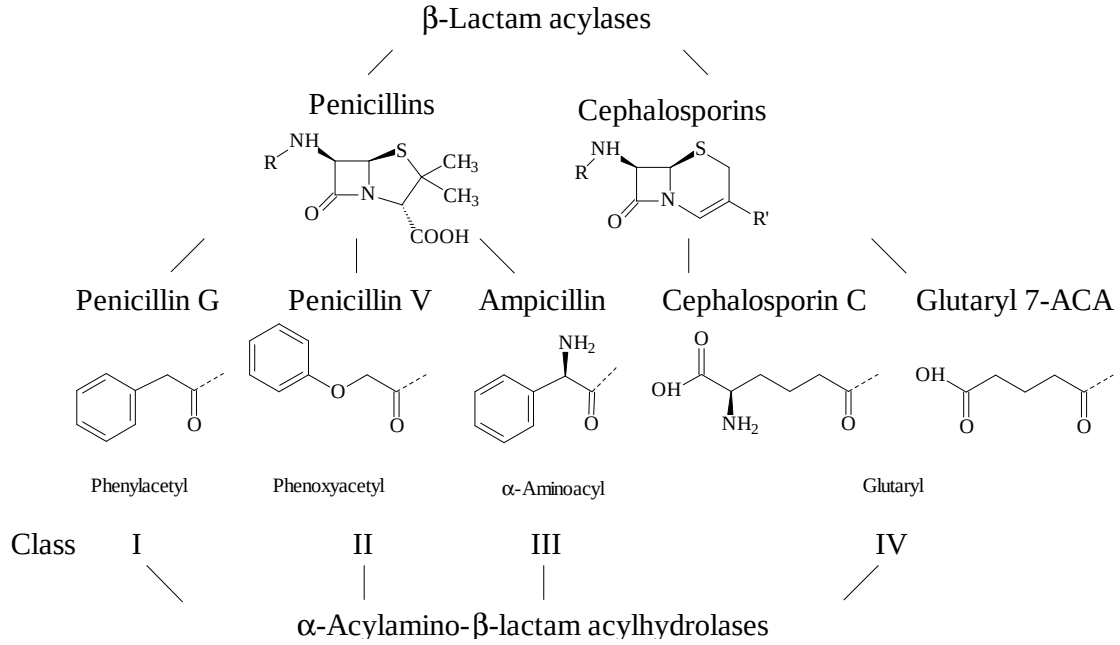


Figure 6. Classical nomenclature of β-lactam acylases. Shown are the two nuclei of the penicillins (6-APA) and cephalosporins (7-ACA derivatives) and subsequently their possible natural side chains (at position R in the nucleus). The classical nomenclature, which is based on the preferred nucleus and subsequently, preferred side chain (R) is shown from the top of the figure. At the bottom of the figure the alternative nomenclature (class I to IV) based on only the preferred side chain is shown.

acylases (Fig. 6).

Penicillin G acylases - Penicillin G acylases (PGAs) prefer penicillins with a phenylacetyl side chain (Fig. 6). One of the first known and industrially used β-lactam acylases is the penicillin acylase from *Escherichia coli* ATCC11105 (PA, EC 3.5.1.11), which is a periplasmic enzyme that is composed of an α- and β-subunit of 24 and 62 kDa, respectively. Cloning of the gene in 1986 showed that the PA is produced as a pre-pro-protein, which consists of a signal sequence, followed by the α-subunit, a spacer peptide, and the β-subunit. Additional studies revealed that the signal sequence is removed upon transport to the periplasm and there the spacer peptide is autocatalytically cleaved from the N-terminal side of the β-subunit and subsequently from the C-terminal side of the α-subunit. This process yields the two separate subunits α and β which are held together by non-covalent bounds (Hewitt et al., 2000; Schumacher et al., 1986).

The mechanism of penicillin acylase involves the formation of an acyl-enzyme intermediate as described for the serine protease chymotrypsin. The crystal structure of PA was solved in 1995 and revealed a single-amino-acid catalytic centre (Duggleby *et al.*, 1995). The hydroxyl group of the serine that is located at the N-terminal end of the β-subunit (βSer1) is activated, via a bridging water molecule, by its own α-amino group (Fig. 7). The serine oxygen attacks the acyl carbon atom of the substrate, forming an oxyanion tetrahedral intermediate (TI_1) that is stabilised via hydrogen bonds by the main-chain amide of Ala69 and the side chain nitrogen of Asn241. Rearrangement of electrons leads to the collapse of the intermediate resulting in release of the leaving group and a covalent acyl-enzyme intermediate. The enzyme is subsequently deacylated by a nucleophile, which leads via a similar tetrahedral intermediate (TI_2) to the free enzyme and the acylation product of the nucleophile. In this way, β-lactam antibiotics can be cleaved to yield the free β-lactam nucleus and the side-chain carboxylic acid when the nucleophilic attack is performed by water (hydrolysis). However, when the acyl donor is an activated synthetic side chain (either an amide or an ester), a nucleophilic attack by a β-lactam nucleus such as 6-APA will yield a semi-synthetic β-lactam antibiotic through a process called aminolysis. Since the discovery of the catalytic mechanism for penicillin acylase, in which an N-terminally located nucleophile is the key residue (Duggleby *et al.*, 1995), several other enzymes have been found to posses a similar mechanism. These enzymes are referred to as the N-terminal nucleophile (Ntn) hydrolases and contain a typical four-layered αββα-core structure (Brannigan *et al.*, 1995).

Homologs of PA are found throughout the whole kingdom of prokaryotes (Arroy *et al.*, 2003). Well-studied PGAs are those from *Kluyvera citrophila* (Barbero *et al.*, 1986; Martin *et al.*, 1991), *Proteus rettgeri* (McDonough *et al.*, 1999), *Alcaligenes faecalis* (Verhaert *et al.*, 1997), *Bacillus megaterium* (Chang and Bennet, 1967; Martin *et al.*, 1995) and *Arthrobacter viscosus*(Konstantinovic *et al.*, 1994; Verhaert *et al.*, 1997). All these PGAs are located in the periplasmic space, expect for the acylases of the later two which are excreted (Gram positive bacteria). As the penicillin acylase from *E. coli*, the other PGAs are composed of two non-identical subunits and have similar molecular weights,

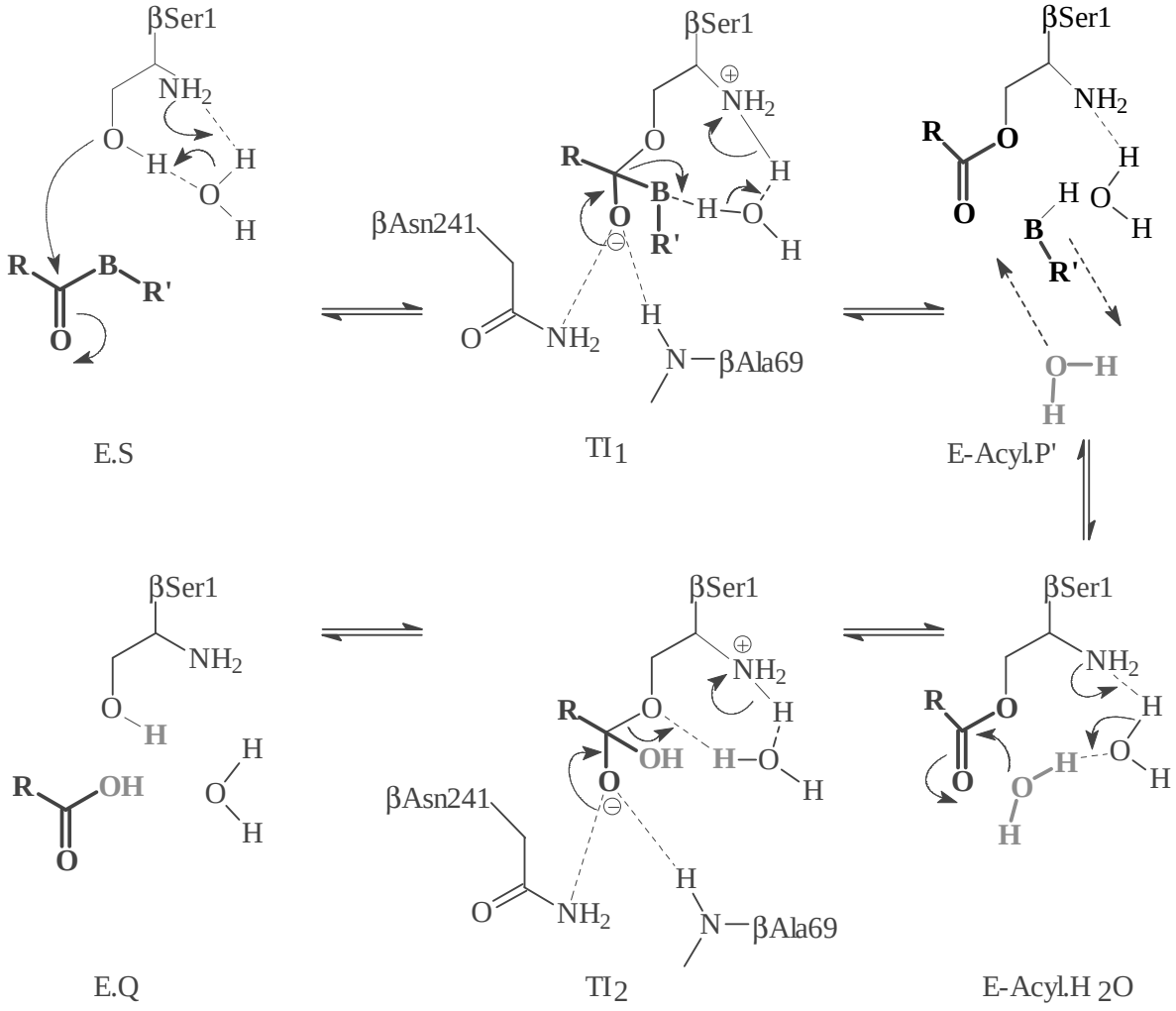


Figure 7. Proposed catalytic mechanism of the Ntn-hydrolase penicillin acylase from *E. coli*. The deacylation by water results in hydrolysis of the substrate [adapted from Duggleby, 1995 #9]. B is either an oxygen (ester) or a nitrogen (amide), R and R' are variable groups. TI, stands for tetrahedral intermediate, either 1 or 2.

sequences show 83 to 29% identity to PA and based on sequence alignments it can be concluded that all these enzymes have an N-terminal catalytic serine and are derived from a precursor protein as described for PA. This was confirmed by the structure of *P. rettgeri*, which indeed showed the typical Ntn-hydrolase structure (McDonough *et al.*, 1999). The PGAs have a similar substrate range although the kinetic parameters can vary among them. For example, the highest affinity (K_m) for penicillin G is found for the *A. faecalis* enzyme (2 µM) (Verhaert *et al.*, 1997) while the acylase of *B. megaterium* ATCC 14945 has a K_m of 4.5 mM (Chang and Bennet, 1967). By comparing the amino acid sequences and the different substrate specificities of the acylases it may be possible to identify residues that determine the catalytic action or biochemical properties of the enzyme. For example, the *A. faecalis* enzyme is more thermostable than *E. coli* PGA, which could be assigned to the unique

presence in the former acylase of two cysteines that form a disulfide bond (Verhaert *et al.*, 1997).

Penicillin V acylases - Penicillin V acylases (PVAs) catalyse the hydrolysis and synthesis of phenoxyacetyl substituted β-lactam antibiotics (Fig. 6). The molecular weights of these acylases vary from 83.2 kDa in *Fusarium* sp. SKF 235 to 140 kDa in *Bacillus sphaericus*, and their subunit composition from monomer to tetramer, respectively. The optimum pH values for the penicillin V acylases range between pH 5.6-8.5, which is lower than found for the PA (pH 6.5-8.5, (Margolin *et al.*, 1980; Schumacher *et al.*, 1986)) and can be an advantage in an industrial process since the chemical degradation of 6-APA is less at lower pH-values (Shewale and Sudhakaran, 1997). The PVAs are mainly produced intracellularly and can be found in many different organisms. Only the gene encoding the penicillin V acylase of *Bacillus sphaericus* (PV, (Olsson *et al.*, 1985)) has been cloned and studied in detail. The amino acid sequence of this β-lactam acylase does not show significant homology with the sequences of known penicillin G acylases. However, the crystal structure of PV reveals an N-terminally located cysteine and the same typical fold as found for PA (Suresh *et al.*, 1999). Therefore it can be concluded that despite its different subunit size and native composition, the penicillin V acylase of *B. sphaericus* belongs to the same structural family as penicillin G acylases.

Ampicillin acylases - Ampicillin acylases are defined as β-lactam acylases that prefer antibiotics with a phenylglycine-derived side chain, such as ampicillin and cephalexin (Fig. 6). In 1972, the first organism to produce an ampicillin acylase was reported (Okachi et al., 1972). Though, based on its substrate range presented in the literature a few years later (Shimizu et al., 1975), it must be concluded that this enzyme from *K. citrophila* belongs to the penicillin G acylase class. In 1973, an ampicillin acylase isolated from *Pseudomonas melanogenum* was described. This enzyme has a completely different substrate range than penicillin G acylases as it catalyzes both the synthesis and hydrolysis of ampicillin but shows no activity with penicillin G or V (Okachi et al., 1973). This substrate range corresponds with that of ealier reported activity of the α-amino acid ester hydrolases (AEHs) by Takahashi et al. (1972). Both the ampicillin acylase and the AEHs need the α-amino group for activity and have subunits of 70-72 kDa. In this thesis these enzymes will be referred to as AEHs and as they are the main subject of this thesis their properties will be described in more detail in a separate paragraph.

Cephalosporin acylases

The cephalosporin acylases (CAs) prefer β-lactam antibiotics with a cephalosporanic acid derived nucleus, such as cephalosporin C and/or glutaryl 7-aminocephalosporanic acid as their substrates. Under physiological conditions, the side chains of glutaryl 7-ACA and cephalosporin C have charged groups. Therefore, cephalosporin acylases (CAs), just as the α-amino acid ester hydrolases but unlike penicillin acylases, can accept β-lactam antibiotics with a charged side chain as their substrate (Fritz-Wolf *et al.*, 2002). The preferred substrate of all cephalosporin acylases appears to be glutaryl 7-ACA. However, cephalosporin acylases that have noticeable activity with cephalosporin C are very interesting from an industrial point of view and are therefore often referred to as a cephalosporin C acylases

(CCA) (Kim *et al*., 2000). The best CCA is found in *Pseudomonas* sp. N176, with a relative activity of 4% on cephalosporin C compared to that on glutaryl 7-ACA. By site-directed mutagenesis its relative activity on cephalosporin C could be further improved to 6% (Ishii *et al*., 1995; Saito *et al*., 1996).

An alternative classification for the CAs has been proposed on basis of gene structure, molecular mass, and enzymatic properties. The ten CAs of which the genes have been cloned were divided into five classes (Li *et al*., 1999). The CAs of class I to IV consist of an α- and β-subunit which vary in size from 16 to 40 and 54 to 22 kDa, respectively. The only class-V CA is the 7 β-(4-carboxybutanamido)-cephalosporanic acid acylase from *Bacillus laterosporus*, which is composed of a single peptide of 70 kDa (Aramori *et al*., 1991b). The amino acid sequences of the enzymes of classes I to III show 15-30% identity with each other and with the different known PAs. The identity with PAs is concentrated at the N-terminal part of the β-subunit, with complete conservation of the N-terminal nucleophile. In addition, it has been found that most of these CAs are subjected to post-translational processing from a preprotein (Aramori *et al*., 1991a; Li *et al*., 1999; Matsuda *et al*., 1987) as described for PA. The similarity between CAs and PAs was confirmed by the recently solved crystal structure of the intracellular heterodimeric CA of *P. diminuta* (class I CA, with α and β subunits of 24 and 63 kDa, respectively) and that of CA from yet another *Pseudomonas* sp. (class was not assigned). Both structures resembled the Ntn-hydrolase fold of the *E. coli* PA structure (Fritz-Wolf *et al*., 2002; Kim *et al*., 2000). Additionally, the structure of CA from *Pseudomonas* sp. N176 (Class III CA) (Kinoshita *et al*., 2000) could be solved by molecular replacement using the *E. coli* PA structure, indicating that the CAs of class I to III belong to the Ntn-hydrolase superfamily.

The cephalosporin acylases of class IV and V show no significant sequence identity with any other β-lactam acylase (Aramori *et al*., 1991b). However, the subunits of class IV CAs are derived from a common precursor protein and their amino acid sequences show significant homology (>30%) with the Ntn-hydrolase γ-glutamyltranspeptidase from *E. coli*, including the conservation of the N-terminally located nucleophilic threonine of the peptidase (Ishiye and Niwa, 1992; Suzuki and Kumagai, 2002), indicating that also the class IV CAs are Ntn-hydrolases.

Thus, in addition to the PAs and PVs, the CAs from class I to IV also belong to the Ntn-hydrolase superfamily, even though in most cases there is little or no sequence identity. Up to now it remains unclear to which structural family the class V glutaryl acylase of *B. laterosporus* belongs.

Ntn-hydrolase superfamily

All the β-lactam acylases structurally and/or genetically characterised thus far have been identified as a member of the Ntn-hydrolase superfamily. The enzymes in this family are activated through autoproteolytic processing catalysed by a residue, which after processing is located at the N-terminus of the β-chain. This residue has two catalytic functions, it is both the nucleophile and the base in catalysis and is either a serine, a threonine or a cysteine. Structurally, the Ntn-hydrolase superfamily was defined as enzymes that show a typical αββα-fold. Up to now, this superfamily consists of markedly

different enzymes with respect to their *in vivo* function, size, subunit composition and substrate range, but they all catalyse amide bond hydrolysis. Enzymes that have been grouped into this superfamily so far include the penicillin G, penicillin V and cephalosporin acylases, proteasomes, a glucosamine 6-phosphate synthase, an aspartylglucosaminidase, L-aminopeptidase-D-Ala-esterase/amidase, class II glutamine amidotransferases, γ-glutamyltranspeptidase and recently, based on sequence similarity with penicillin V acylases, the peptidase family U34 (which includes enzyme such as choloylglycine hydrolases and isopenicillin N acyltransfereses) was added (Kim *et al*., 2000; Oinonen and Rouvinen, 2000; Pei and Grishin, 2003; Suzuki and Kumagai, 2002).

Classification of β-lactam acylases

Although old-fashioned, the nomenclature according to the preferred β-lactam antibiotic substrate coincides quite nicely with the separate

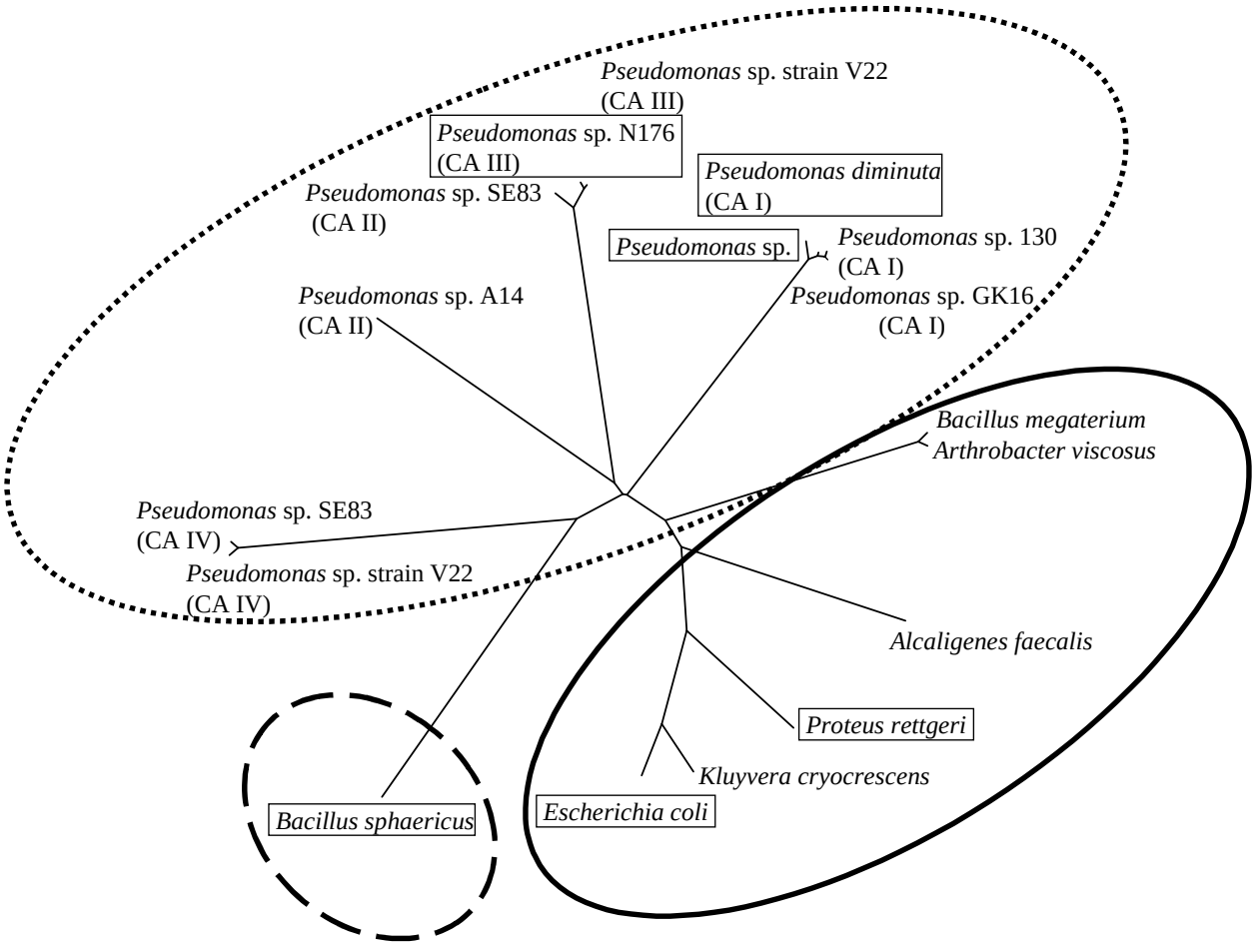


Figure 8. Dendrogram of representative Ntn-hydrolase β-lactam acylases. The acylases are indicated by their natural host species. The bold circles indicate the classical classification based on substrate specificity: —, penicillin G acylases; ---, cephalosporin acylases; and, — — , penicillin V acylase. Boxed species refer to acylases of which the structure was solved. As no sequence or structural similarity with other β-lactam acylases has been found for the glutaryl acylase from *B. laterosporus* (class-V CA) this enzyme was not included in the dendrogram. All the β-lactam acylases indicated in the dendrogram are structurally related as they all belong to the Ntn-hydrolase superfamily. The tree was created using Clustal W and TreeView.

groups found in the classification based on the similarity of the amino acid sequences of the different β-lactam acylases (Fig. 8). In the latter classification, the penicillin G, penicillin V and cephalosporin acylases form separate groups as well. The CAs, however, are not confined to one specific group but are divided in three separate clusters, which closely resembles the alternative classification proposed for the CA described above. The fact that according to their three-dimensional structure or sequence analysis, both members of penicillin and cephalosporin acylase classes belong to the Ntn-hydrolase superfamily indicates that there is an extended evolutionary relationship among the different β-lactam acylases. The question is whether the class-V CA from *B. laterosporus* and the ampicillin acylases or better the α-amino acid ester hydrolases (of which no amino acid sequence is available) also belong to this structural family. The results presented further in this thesis will show that this is not the case but that they instead belong to another superfamily of hydrolytic proteins.

α-AMINO ADIC ESTER HYDROLASES

In search for organisms able to synthesise β-lactam antibiotics that have a free amino group in their side chain, Takahashi *et al.* reported the ability of some bacteria to synthesise unnatural cephalosporins by the acylation of 7-aminocephem compounds with α-amino acid esters in 1972 (Takahashi *et al.*, 1972). The enzymes responsible for this activity were named α-amino acid ester hydrolases (AEHs) as i) their substrate range was limited to acyl donors with an α-amino group, ii) they preferred esters over amides and iii) because enzymes that can catalyse both transfer and hydrolysis reactions are referred to as hydrolases according to the rules for enzyme nomenclature (Takahashi *et al.*, 1974). In the period from their discovery up to 1990, the α-amino acid ester hydrolases of five organisms had been purified and their substrate specificity was determined: *Xanthomonas citri* (α_4, subunit size 72 kDa) (Kato *et al.*, 1980a), *X. rubrilineans* (α_4, subunit size 72 kDa) (Krest'ianova *et al.*, 1990), *X.* sp. (α_4, subunit size 70 kDa) (Blinkovsky and Markaryan, 1993), *Acetobacter turbidans* (α_2/β_2, subunit size 70/72 kDa, respectively) (Ryu and Ryu, 1987) and *P. melanogenum* (α_2, subunit size 72 kDa) (Kim and Byun, 1990b). All these AEHs show an absolute need for the α-amino group, which makes them very suitable for the synthesis of β-lactam antibiotics with a phenylglycine side chain, such as ampicillin or cephalexin, and derivatives thereof, such as amoxicillin and cephadroxil (Fig. 4). Unfortunately, they do not exhibit absolute stereospecificity as they hydrolyse esters of both D- and L- amino acids. However, the preference for a specific configuration of a particular amino acid has been observed (Fernandez-Lafuente *et al.*, 2001). The pH optimum of the AEHs varies between pH 6.0 – 6.5 and the optimal temperatures vary within the range of 35-45 °C. The preferred β-lactam antibiotic substrate is cephalexin (Kato *et al.*, 1980b; Kim and Byun, 1990b; Takahashi *et al.*, 1974). Therefore, according to the classical β-lactam acylase nomenclature, the AEHs should be classified as cephalexin acylases (Kato *et al.*, 1980b; Nam *et al.*, 2001; Ryu and Ryu, 1988). Alternatively, they belong to class III of the α-acylamino-β-lactam acylhydrolases.

Catalytic mechanism

The AEHs catalyse two reactions, the N-acylation of β-lactam nuclei with α-amino acid groups to give the corresponding semi-synthetic β-lactam antibiotic, and the hydrolysis of α-amino acid esters or β-lactam antibiotics (Fig. 9). Based on the observations that i) the hydrolytic and transfer reactions showed identical substrate.specificities and displayed the same pH-optimum; ii) the addition of nucleus diminished the hydrolysis rate of the activated ester, but with the corresponding amount of β-lactam antibiotic formed, the overall ester consumption remained unchanged and iii) the ratio of hydrolysis versus transfer rate did not depend on the ester moiety of the acyl donor, it has been concluded that the AEHs catalyse their reactions through a common acyl-enzyme intermediate (Blinkovsky and Markaryan, 1993; Kato, 1980; Takahashi *et al.*, 1974). The acyl-enzyme mechanism corresponds to the mechanism described penicillin acylases and for peptide hydrolysis by serine hydrolases. In such a mechanism, the substrate first associates with the enzyme to form a noncovalent enzyme-substrate complex (Michaelis-Menten complex) followed by the acylation of the active site serine to give the acyl-enzyme intermediate (Fig. 10). This acyl-enzyme is attacked by a nucleophile (AcA, acyl acceptor) to give the enzyme-product complex from which the product is released. In case of the AEHs and PAs the acyl side chain can be transferred to water or to amine nucleophiles (like β-lactam nuclei) resulting in hydrolysis or formation of a semi-synthetic β-lactam antibiotic, respectively (Kato, 1980; Takahashi *et al.*, 1974).

Catalytic triad

Despite the similarity of the proposed mechanism of the AEHs with that described for serine hydrolases (such as PA), no inhibition of

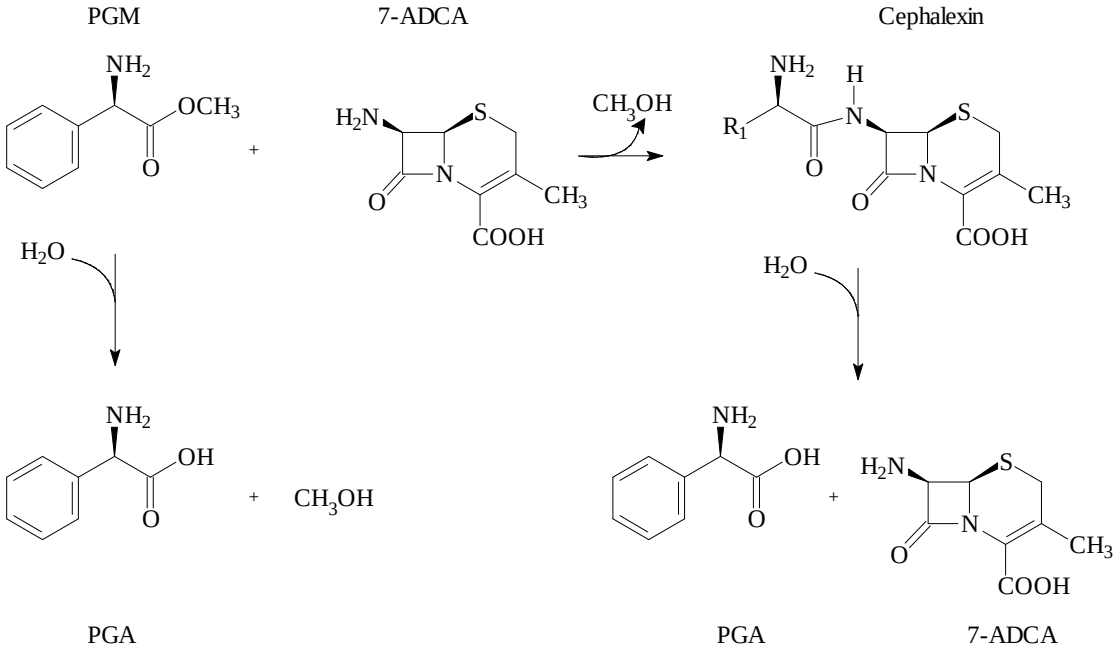


Figure 9. Cephalexin synthesis and hydrolysis reactions performed by AEHs. PGM, phenylglycine methyl ester; 7-ADCA, 7-aminodesacetoxycephalosporanic acid and PGA, phenylglycine acetic acid.

AEH activity by serine hydrolase inhibitors like phenylmethylsulfonyl fluoride (PMSF) or diisopropylphosphorylfluoride (DIPF) was found (Blinkovsky and Markaryan, 1993; Ryu and Ryu, 1988). Chemical modification studies on the AEH from *A. turbidans* indicated the importance of a histidine residue (Ryu and Ryu, 1988), which was confirmed by similar studies on the enzyme of *P. melanogenum* (Kim and Byun, 1990a). Additionally, for the enzyme of *X. citri* it was found that both an amino group and a carboxylic acid group were essential for catalysis (Nam *et al*., 1985). Therefore, it is likely that in the AEHs multiple amino acids are essential in catalysis which might indicate a different mechanism than found for other β-lactam acylases.

In the proteolytic enzyme chymotrypsin, the first existence of three active site residues, a nucleophile, a base and an acid, present in a configuration now known as the catalytic triad was described. In chymotrypsin these residues are a serine, a histidine, and an aspartate, respectively. Nowadays, many variants of this classical catalytic triad are found in proteolytic and non-proteolytic enzymes (Dodson and Wlodawer, 1998). For example, in a protease from the hepatitis A picornavirus the nucleophilic serine is replaced by a cysteine residue (Dodson and Wlodawer, 1998) and in the haloalkane dehalogenase from *Xanthobacter autotrophicus* by an aspartate (Verschueren *et al*., 1993). In acetylcholine esterase from *Torpedo californica*, the acid is replaced by the similar glutamate and in an esterase from *Streptomyces scabies* it is replaced by two main chain carbonyl oxygens (Wei *et al*., 1995). In β-lactamases, the generally conserved histidine (base) is replaced by a lysine (Dubus *et al*., 1994). Sometimes an amino acid has a double role. For example, in the Ntn-hydrolase family the OH and α-NH_2 functions of the N-terminal serine serve as a nucleophile and a base, respectively (Duggleby *et al*., 1995). Alternatively, in some proteases a catalytic tetrad rather than a triad is conserved, in which the fourth residue is either a serine or a cysteine that stabilizes the other catalytic residues (Krem and Di Cera, 2001).

The catalytic triad and variants thereof are present in many different structural folds. Examples of protein folds that provide a scaffold for a classical catalytic triad are the trypsin- and subtilisin-like serine protease-, cysteine proteinase-, flavodoxin-like- and the α/β-hydrolase-fold enzymes. Further inhibition studies and the three-dimensional structure are needed to gain more insight into the catalytic mechanism of the AEHs.

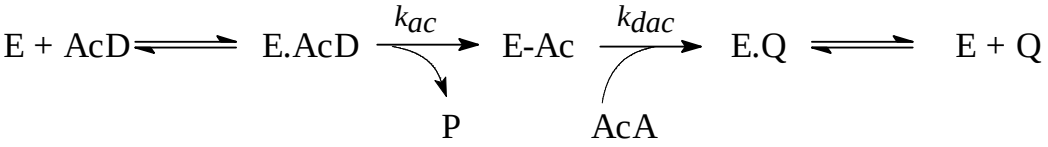


Figure 10. The proposed reaction scheme for AEH involving an acyl-enzyme intermediate. In the scheme E is short for enzyme and represents the AEHs; AcD, acyl donor, either an α-amino ester or an α-amino amide; P, the first product, either an alcohol or an amide; E-Ac, acyl-enzyme intermediate; AcA, acyl acceptor, amide or water; Q, transfer product, i.e. the antibiotic (aminolysis) or acid (hydrolysis); k_{ac}, acylation constant and k_{dac}, deacylation constant.

Cloning of the AEH encoding genes

For an extended detailed analysis of the AEHs, large quantities of enzyme are needed which can easily be obtained when the encoding genes are available for overexpression. One attempt to clone an AEH-encoding gene was under taken by Nam *et al.* using an immunochemical detection method. Therefore, two λ gt11 genomic libraries of *A. turbidans* ATCC 9325 were made and screened with a polyclonal mouse antibody against purified *A. turbidans* AEH (Nam and Ryu, 1988). Two positive clones were obtained, one producing a protein of 108 kDa and the other one of 180 kDa. From restriction analysis, Nam *et al.* concluded that some parts of the construct and insert DNA were deleted during the replication in the host cells, which hampered the cloning of the full gene. In their conclusion they stated that they would use the insert DNA of the positive clones as probes to clone the full gene. However, up to now, no report has been made of the successful cloning of the *A. turbidans* AEH gene by Nam *et al*. Another attempt to clone an AEH was undertaken by Alonso and García *et al.* (Alonso and García, 1996). They constructed a genomic library of the total DNA of *X. campestris* pv. *citri* IFO 3835 in pBR322 and transformed it to the leucine deficient host *E. coli* HB101. The selection procedure was based on the auxotrophic complementation of the host, growing it on a minimal medium containing D-alanine-L-leucine. It was expected that when a clone harboured and expressed the gene encoding the AEH, it could hydrolyse this dipeptide, releasing L-leucine required for growth. In this way, one positive clone was obtained of which the extract, unfortunately, was not able to hydrolyse ampicillin. Sequence analysis and homology searches showed that the cloned gene encoded a proline iminopeptidase. Thus, the two cloning attempts described in literature thus far, which were both based on screening for expressed enzyme, were unsuccessful. This propably hampered the introduction into an industrial semi-synthetic β-lactam synthesis process for which they have promising properties

BIOCATALYTIC PRODUCTION OF SEMI-SYNTHETIC β-LACTAM ANTIBIOTICS

Nowadays, the β-lactam antibiotics penicillin G and V are fermentatively produced at an estimated annual market volume of 33,000 tons world-wide using *Penicillium* strains. About 10,000 tons of this is used as a starting material for semi-synthetic β-lactam antibiotics (Elander, 2003). The production of the latter comprizes two processes, the hydrolysis of the fermentative β-lactam antibiotic and the condensation reaction of the obtained free β-lactam nucleus (expanded to 7-ADCA or not) with a new side chain. The enzymes described above, especially PA from *E. coli*, and the AEHs are interesting from an industrial point of view as they can be used in the development of green alternatives for the chemical routes in the production of semi-synthetic β-lactam antibiotics.

Hydrolysis of fermentative β-lactam antibiotics

Chemical hydrolysis - Although the enzymatic removal of the side chain of fermentative penicillins was already demonstrated in 1960, it was not implemented in industry because at that time enzymes were considered difficult to work with. Additionally, they were hard to obtain in large quantities and difficult to recover, making the use of enzymes expensive.

Therefore, alternative chemical routes were developed for the isolation of β-lactam nuclei from fermentatively produced β-lactam antibiotics (Verweij and Vroom, 1993). The chemical hydrolysis is complicated due to the high reactivity of the β-lactam bond. However, after protecting the carboxyl group of the β-lactam nucleus with silyl chemistry, the side chain amide bond can be selectively cleaved in the presence of phosphorus pentachloride and alcohol at low temperatures. The development of this so-called 'Delft cleavage' led to a very efficient and inexpensive one-pot synthesis of 6-APA. This procedure could be applied efficiently for the chemical cleavage of cephalosporin C as well, resulting in the availability of the 7-ACA nucleus for the production of semi-synthetic cephalosporins (Bruggink, 2001; Verweij and Vroom, 1993).

Enzymatic hydrolysis -The development of robust and immobilised biocatalyst systems (enabling recycling) allowed the introduction of an economically feasible biocatalytic process for the hydrolysis of fermentative penicillins using the penicillin G or V acylases in the mid 1980s (Fig. 11) (Shewale and Sudhakaran, 1997; Van de Sandt and De Vroom, 2000). The cloning of the genes encoding PVAs and PGAs (see above) enabled efficient production of large quantities of enzyme in overexpression systems contributing to the success of the introduction of these biocatalysts. The use of enzymes allowed the hydrolytic process to be performed in water, at ambient temperature, and with only a titrant as auxiliary chemical, which led to a 5-fold reduction of the produced waste volume compared to the chemical route (Van de Sandt and De Vroom, 2000).

Also in the production of cephalosporin related β-lactam nuclei biocatalysis was introduced. A two-step enzymatic process replaced the chemical hydrolysis of cephalosporin C to yield 7-ACA, since no enzyme was available to directly deacylate cephalosporin C. A D-amino acid oxidase is used in the conversion of the α-aminoadipyl to a glutaryl side chain, which is subsequently hydrolysed by a glutaryl 7-ACA acylase to yield 7-ACA (Fig. 11). The other important cephalosporin nucleus is 7-aminodesacetoxycephalosporanic acid (7-ADCA, Fig. 11, (5)). Chemically, this nucleus was produced from penicillin G by chemical ring expansion using peracetic acid, hydrogen bromide and pyridine, converting penicillin G into cephalosporin G, and subsequent chemical deacylation (Delft cleavage). In a green alternative, the deacylation of cephalosporin G by PA was introduced (Fig. 11, (3) to (5)).

A completely green route to 7-ADCA can be obtained by introduction of a new fermentation process using a *Penicillium chrysogenum* strain expressing the expandase gene from *Streptomyces clavuligerus*. Experiments showed the successful production of adipoyl-7-ADCA (Robin *et al.*, 2001) of which the side chain can easily be removed by a glutaryl acylase related enzyme. The industry has started with the implementation of this fully biosynthetic manufacturing process (Bruggink, 2001). Alternatively, the expandase gene from *Streptomyces clavuligerus* can replace the gene encoding the bifunctional expandase/hydroxylase activity in the cephalosporin C production strain *Acremonium chrysogenum*, resulting in the production of desacetoxycephalosporin C. Subsequent deacylation by D-amino acid oxidase and glutaryl

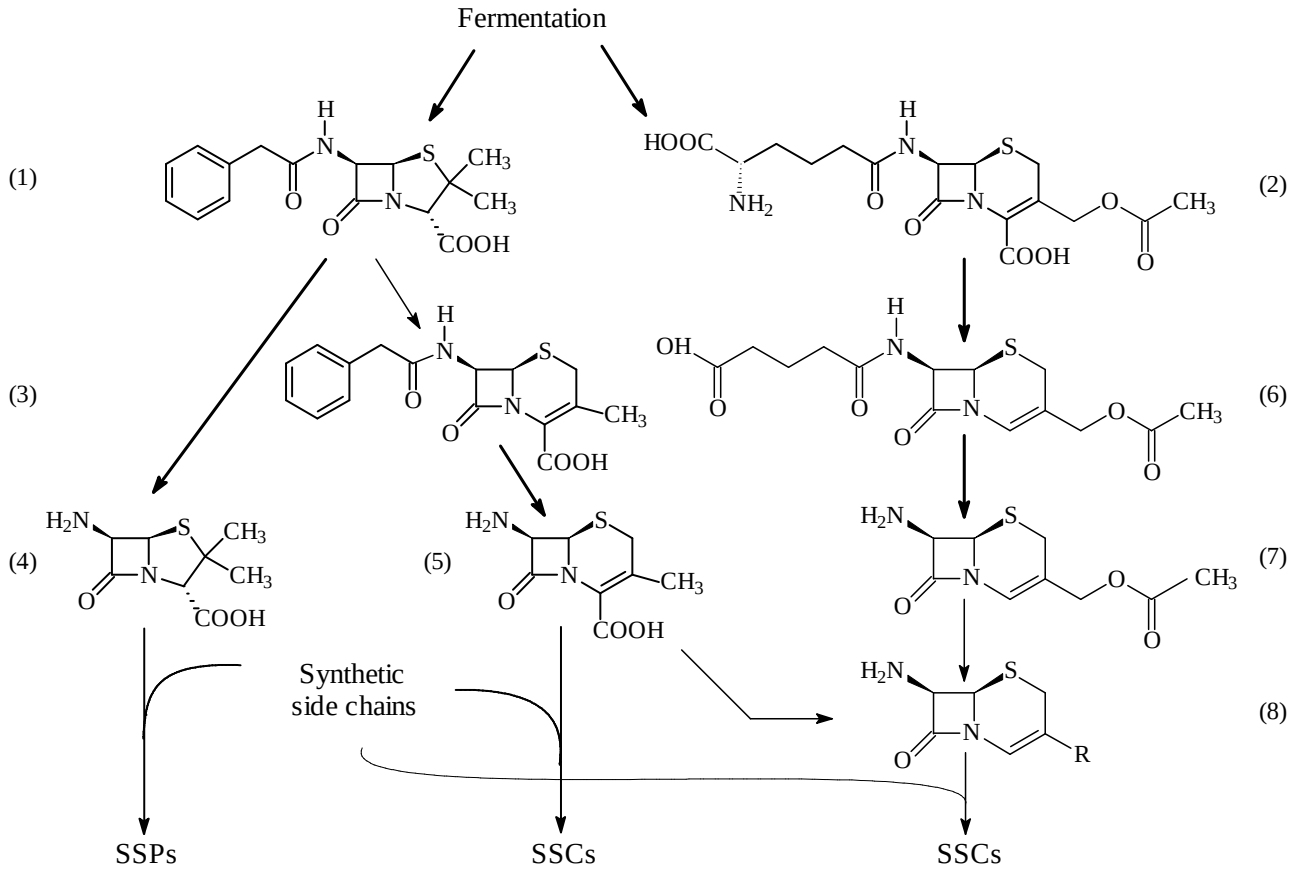


Figure 11. Production of semi-synthetic β-lactam antibiotics. (1), Penicillin G; (2), cephalosporin C; (3), cephalosporin G; (4), 6-APA; (5), 7-ADCA; (6), glutaryl-7-aminocephalosporanic acid; (7), 7-ACA and (8) Cephem nuclei with different substituents. Bold arrows indicate routes dominated by biocatalysis within industry. The other routes are under development to reach optimal results with biocatalysis.

acylase yields 7-ADCA (Velasco *et al.*, 2000).

Synthesis of semi-synthetic β-lactam antibiotics

Chemical synthesis - To couple synthetic side chains to the free β-lactam nuclei two major chemical routes are used, the Schotten-Baumann condensation with acid chloride and the Dane salt method. In the robust acid chloride process, the phenylglycine side chain is activated by making its acid chloride hydrochloride that is subsequently coupled to a silyl-protected 6-APA to obtain ampicillin (Fig 12, A). In the Dane salt method the side chain is coupled via its amine group to a β-dicarbonyl compound. The resulting anhydride (the Dane salt is subsequently converted to a mixed activated side chain) that can directly be coupled to the free β-lactam nucleus in the presence of an organic base (Fig. 12, B) (Bruggink, 2001). Unfortunately, both methods need low temperatures and organic solvents resulting in a considerable burden for the environment.

Enzymatic synthesis - To shift from chemical to an enzyme-catalysed synthesis of semi-synthetic β-lactam antibiotics, two types of enzymes have been explored for their

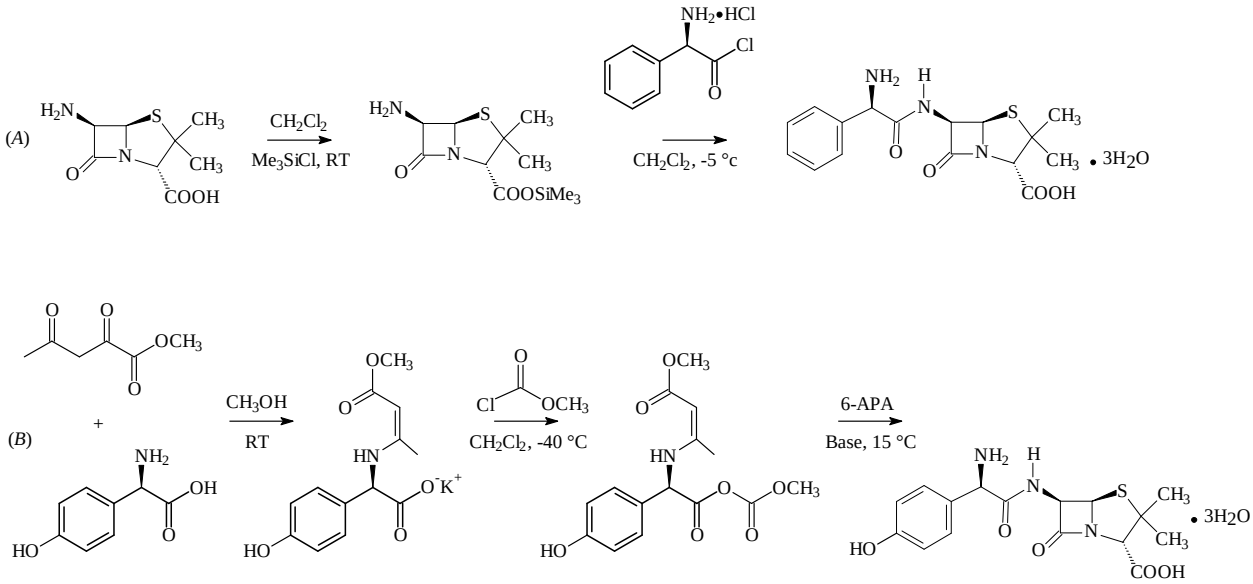


Figure 12. Chemical routes to semi-synthetic β-lactam antibiotics. (*A*) Schotten-Baumann condensation with the acid chloride of phenylglycine producing ampicillin, (*B*) Dane salt method for the synthesis of amoxicillin.

applicability, the penicillin acylase from *E. coli* and the α-amino acid ester hydrolases (AEHs) (Bruggink, 2001; Bruggink *et al*., 1998). These enzymes can couple a synthetic side chain to the free β-lactam nucleus producing a semi-synthetic β-lactam antibiotic in a thermodynamically or kinetically controlled process.

Thermodynamic coupling - In a thermodynamic coupling, the semi-synthetic β-lactam antibiotic is formed by an enzyme-catalysed condensation between the free carboxylic acid and the β-lactam nucleus. The enzyme influences the rate at which the equilibrium is reached and the thermodynamic equilibrium between the products and reactants under the conditions used determines the maximum product accumulation. Since the product concentration at equilibrium is often low (Blinkovsky and Markaryan, 1993; Schroën et al., 1999), this reaction is considered feasible only when the equilibrium can be shifted towards synthesis by product removal, for example by crystallisation or complexation (Schroën et al., 1999). For the production of α-amino substituted β-lactam antibiotics, phenylglycine (PG) and derivatives thereof needs to be coupled to the nucleus. In de pH-range (4-9) where the enzymes are active, the amino group of PG is mainly present in its charged form (Blinkovsky and Markaryan, 1993; Schroën et al., 1999). Penicillin acylase from *E. coli* does not accept a charged amino group (Margolin et al., 1980), while the AEH of *X. citri* does (Blinkovsky and Markaryan, 1993). Unfortunately, thermodynamic coupling of PG to 7-ADCA by AEH without product complexation or removal resulted in only small amounts of antibiotic due to unfavourable thermodynamic conditions (Blinkovsky and Markaryan, 1993; Schroën et al., 1999).

Kinetic coupling - In the kinetically controlled process an activated substrate is used (amide or ester) to acylate the enzyme. Subsequent transfer of the acyl group to an accepting nucleophile leads to an acylated

product. The kinetic properties of the enzyme and the conversion process allow the transient accumulation of high levels of synthesis products (Q_{max}) before equilibrium is reached. However, unwanted enzyme-catalysed reactions take place as well, like the hydrolysis of the activated acyl donor and the hydrolysis of the condensation product. The level of Q_{max} is determined by the kinetic parameters of the enzyme, and is independent of the enzyme concentration. Another parameter used to evaluate the synthesis reaction is the ratio between the condensation and hydrolysis product, the so-called synthesis/hydrolysis (S/H) ratio. In this study, the S/H ratio is determined from the product concentrations at Q_{max} (S/H_{max}) and from the formation rates in the beginning of a synthesis reaction (S/H). Both these ratios are determined by the enzyme and reaction conditions like temperature, ionic strength and pH (Kasche, 1986).

AEH in the synthesis of semi-synthetic β-lactam antibiotics

The catalytic properties and substrate range of AEHs have many properties that make them interesting candidates for application in the kinetically controlled synthesis of α-amino substituted semi-synthetic β-lactam antibiotics. For example, due to their low affinity for amides, low product (amide) hydrolysis is expected which might lead to a high level of product accumulation. Furthermore, the AEHs are not inhibited by phenylacetic acid (PAA) (Blinkovsky and Markaryan, 1993), which may be present in small amounts in 6-APA that is produced from penicillin G. If PA is used to couple 6-APA to synthetic side chains, the starting materials must be free of any traces of PAA, since the *E. coli* PA is strongly inhibited by it. The use of AEHs would eliminate the need for purification of the β-lactam nuclei and any losses accompanying it. To reduce the number of operations and decrease the losses of 6-APA accompanying them in a synthesis process, a one-pot synthesis in which the enzymatic hydrolysis of penicillin G and the coupling of the obtained nucleus to synthetic side chains take place in one reaction vessel is very attractive. Due to the high concentrations of PAA that accumulate, it is unfavourable to use *E. coli* PA in this process. However, the lack of inhibition by PAA of the AEHs, might make these enzymes an interesting alternative. The applicability of AEH from *A. turbidans* to synthesise amoxicillin in the presence of PAA has been demonstrated, although removal of PAA and remaining penicillin G up to a certain level is preferred to reduce the ionic strength and increase the yield (Diender, 2001). Another advantage of the AEHs is that they have a high preference for the D-isomer of phenylglycine methyl ester (D-PGM, the activated side chain) in the acylation reaction. Therefore, synthesis reactions can be carried out starting from a racemic mixture of phenylglycine methyl ester (Fernandez-Lafuente *et al*., 2001), making a resolution process to produce enantiopure phenylglycine methyl ester unnecessary (Bruggink, 2001). Although, from an industrial point of view introducing racemic side chain donors in the condensation reaction is not interesting since it would lead to a very complicated downstream processing, eventually nullifying the benefits (Bruggink *et al*., 1998). Finally, the lower pH optimum of the AEHs as compared to PAs is advantageous since the precursors and products of β-lactam antibiotic synthesis are more stable at lower pH (Schroën *et al*., 1999; Shewale and Sudhakaran, 1997).

Additionally, the ability of the AEHs to synthesise ampicillin from D-PGM with high efficiency at low temperatures (5 °C) is very beneficial, as the low temperature contributes to the stability of the precursors and formed product, reducing (unproductive) losses.

Although the AEHs seem to have many advantages over PA, penicillin acylases were first introduced in the kinetically controlled production of semi-synthetic β-lactam antibiotics (cephalexin) (Bruggink *et al.*, 1998). This was mainly due to the improved gelatin-based immobilisation of PA, which had been optimised for the highest molar ratio of product to hydrolysis product at 10% conversion (S/H ratio). Additionally, the gene encoding PA was cloned and its expression had been optimised providing in the need for large quantities of enzyme. Furthermore, conditions for use in an industrial process had already been established with the hydrolysis of the fermentative β-lactam antibiotics. The introduction of biocatalysis in the coupling process (Fig. 11) reduced the amount of organic reagents and solvents needed with a factor 5 and made the use of liquid nitrogen and halogenated solvents superfluous (Bruggink, 2001). The conversion of a chemical to an enzymatic coupling processes for other semi-synthetic β-lactam antibiotics, such as ampicillin, amoxicillin, cefaclor and cefadroxil, needs further development to allow integration in the industrial production.

Thus, introduction of AEHs in biocatalysis has very likely been hampered by the limited availability of the enzymes, caused by the low expression level of the AEHs in the natural hosts and the absence of cloned genes and overexpression systems. Furthermore, a detailed kinetic and mechanistic insight, which can provide a rationale for process optimisation and enzyme engineering, has been lacking so far.

SCOPE AND OUTLINE OF THIS THESIS

The work presented in this thesis was performed as part of the so-called Clusterproject Catalysis in Fine Chemistry. In the Clusterproject universities supported by industry studied the possible introduction or improvement of biocatalytic and non-biocatalytic routes in the production of β-lactam antibiotics from many different angles. The disciplines that were represented varied from process technology and organic synthesis to enzymology and structural biology. The research of which the results are described in this thesis involved the search for and characterisation of enzymes that can be used for the preparation of α-amino substituted semi-synthetic β-lactam antibiotics. To find the best enzyme for this process we first compared various strains expressing different classes of β-lactam acylases for their capacity to synthesis ampicillin from phenylglycine ester or amide and the 6-APA nucleus (*Chapter 2*). The strains that expressed α-amino acid ester hydrolases showed the best biocatalytic properties and were selected for further studies. As mentioned before, AEHs were not easily available due to low expression levels in the wild-type organisms and complicated purification procedures. To facilitate further studies such as the determination of the catalytic mechanism and X-ray structure, it was decided to clone the genes encoding the AEHs. To that purpose, genome libraries of the total DNA from each organism were made (*Chapter 2, 3 and 5*). We attempted to locate the genes encoding the AEHs in the separate libraries by several activity assays (*Chapter 2*), but unfortunately this was not

successful. Probably, the enzymes were not correctly expressed in the library host and it was decided to purify the AEHs from the wild-type organisms. From the pure enzymes, parts of the amino acid sequences were determined, which in turn were used to design a specific DNA probe enabling the location of the genes in the library via DNA hybridisation. In this way the genes from *A. turbidans* ATCC 9325 and *X. citri* IFO 3835 were cloned and characterised (*Chapter 3* and *5*, respectively).

In *Chapter 4* we provide more insight in the catalytic mechanisms of the AEHs through labelling with *p*-nitrophenyl *p'*-guanidino benzoate (*p*-NPGB) and the subsequent identification of the catalytic nucleophile of the AEH from *A. turbidans* by mass spectrometry. Additional sequence analysis, homology searches and site-directed mutagenesis lead to the identification of a catalytic triad in the AEHs. Database searches with the cloned AEHs identified a new class of β-lactam acylases of more than 7 putative AEHs that showed at least 60% identity. The catalytic mechanism of the AEHs was further elucidated by the crystallographic studies on the AEH of *X. citri* (*Chapter 5*). The structure revealed a rather complex but elegant system of three separate domains comprising one subunit. By combining some experimentally determined features with the structure, insight was obtained in the catalytic mechanism of the AEHs.

In *Chapter 6* we describe the first site-directed mutagenesis experiments of the AEH from *A. turbidans* to improve its biocatalytic performance. These studies resulted in two mutants with improved properties. *Chapter 7* presents the main conclusions of this thesis and will elaborate on future research needed to gain more insight in the structure-function relationship of this new class of β-lactam acylases.

2

Screening of β-lactam acylases for ampicillin synthesis

Jolanda J. Polderman-Tijmes, René Floris and Dick B. Janssen

ABSTRACT

Enzymes possessing β-lactam acylase activity can catalyze the cleavage and synthesis of β-lactam antibiotics by hydrolyzing or forming the amide bond that connects the side chain and the β-lactam nucleus in these antibiotics. To find the best enzyme for the synthesis of ampicillin we compared twelve microbial enzymes with β-lactam acylase activity towards various substrate classes. The α-amino acid ester hydrolases expressed by *Acetobacter turbidans*, *Xanthomonas citri* and *Acetobacter pasteurianus* showed a high affinity for α-amino substituted substrates and gave the highest level of product accumulation in ampicillin synthesis. They also showed low product hydrolysis and could perform synthesis at low pH-values, which is beneficial for the stability of the products and precursors. These properties indicate that α-amino acid ester hydrolases may be attractive for biocatalytic steps in the production of ampicillin or other α-amino substituted semi-synthetic β-lactam antibiotics.

INTRODUCTION

A β-lactam antibiotic consists of the antibacterial β-lactam nucleus and a so-called side chain. These moieties are connected via an amide bond. The β-lactam antibiotics are grouped in penicillins, which have a 6-aminopenicillanic acid nucleus, and cephalosporins, which have a 7-amino-cephalosporanic acid β-lactam nucleus. The most widespread mechanism that causes bacteria to be resistant to a particular β-lactam antibiotic is the hydrolysis of the β-lactam peptide bond by a β-lactamase. The susceptibility of a certain antibiotic to β-lactamase activity is strongly influenced by the nature of the side chain. Therefore, altering the side chain of a fermentatively produced β-lactam antibiotic can yield derivatives that are not readily inactivated by β-lactamases. Additionally, changing the side chain can alter the antibacterial spectrum and the pharmacological properties of a β-lactam antibiotic. These observations led to the introduction of the semi-synthetic β-lactam antibiotics, which consist of a β-lactam nucleus derived from an antibiotic produced by fermentation, and a modified synthetic side chain. For example, in the semi-synthetic β-lactam antibiotic ampicillin the phenylacetic acid side chain from penicillin G is replaced by the synthetic side chain phenylglycine. The introduction of the α-amino group makes this β-lactam antibiotic more stable at acidic pH and more soluble in water than penicillin G, which is beneficial for oral administration (Nicholas *et al.*, 1995). The large flexibility of their effective structure has made the β-lactam antibiotics the most successful type of drugs for the treatment of infectious diseases up to now.

The manufacturing process of semi-synthetic β-lactam antibiotics can be divided into two steps: i) the fermentative production of a β-lactam antibiotic and its hydrolysis; and ii) the coupling of a synthetic side chain to the free β-lactam nucleus. In both steps enzymes with β-lactam acylase activity are or can potentially be used to create an efficient and environmentally benign process.

Enzymes with β-lactam acylase activity have been found in bacteria, actinomycetes, yeast and fungi. Based on the preferred β-lactam

Table 1. The selected organisms expressing a β-lactam acylase.

Organism	Enzyme	Loc.	Subunits	Gene	Score (%)	$pH_{opt.}$	pI	pH-range	Ref.
Xanthomonas citri IFO 3835	AEH	I	$(\alpha)_4$ 72 kDa	-		6.4	7.8	5.5-7.5	Kato, 1980; Kato and Kakinuma, 1980; Kato *et al*., 1980a; Kato *et al*., 1980b
Xanthomonas maltophilia IFO 12020	AEH	I	$(\alpha)_2$ 70 kDa	-		6.0	7.2	5.5-6.5	Kim and Byun, 1990
Xanthomonas sp.•	AEH	I	$(\alpha)_4$ 70 kDa	-		6.4	7.6	4.5-8.5	Blinkovsky and Markaryan, 1993
Xanthomonas rubrillineans•	AEH	I	$(\alpha)_4$ 70-72 kDa	-		6.5	6.8	4.0-8.0	Krest'ianova *et al*., 1990
Acetobacter turbidans ATCC 9325	AEH	I	$(\alpha)_2(\beta)_2$ 70/72 kDa	-		6.0-6.2	5.8	5.2-7.5	Takahashi *et al*., 1974
Acetobacter pasteurianus	AEH	n.d.	n.d.	-		n.d.	n.d.	n.d.	Takahashi *et al*., 1972
Achromobacter NRRL B-5393	AEH	n.d.	n.d.	-		n.d.	n.d.	n.d.	Fujii *et al*., 1976
Escherichia coli ATCC 11105	PA	P	αβ 23/63 kDa	+	100	6.5-8.5	6.5	5.0-8.0	Margolin *et al*., 1980; Schumacher *et al*., 1986
Kluyvera cryocrescens ATCC 21285	PA	P	αβ 24/62 kDa	+	85	6.5	6.8	4.0-8.0	Barbero *et al*., 1986
Bacillus megaterium ATCC 14945	PA	E	αβ 24/62 kDa	+	29	8.0-9.0	n.d.	n.d.	Chang and Bennet, 1967; Meevootisom and Saunders, 1987
Arthrobacter viscosus DSM 20159	PA	E	αβ 24/62 kDa	+	28	n.d.	n.d.	n.d.	Konstantinovic *et al*., 1994
Pseudomonas sp. SE83†	GA	C	αβ 24/40 kDa	+	19	n.d.	n.d.	n.d.	Matsuda *et al*., 1987
Pseudomonas diminuta N176	CA	C	αβ 26/58 kDa	+	15	9	4.5	6.0-10	Aramori *et al*., 1991a; Aramori *et al*., 1992
Bacillus sphaericus ATCC 14577	PVA	I	$(\alpha)_4$ 35 kDa	+	10	5.8	4.8	4.5-7.0	Olsson *et al*., 1985

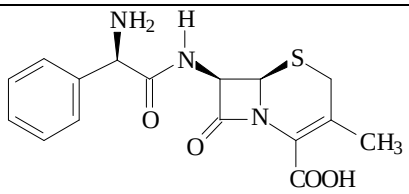
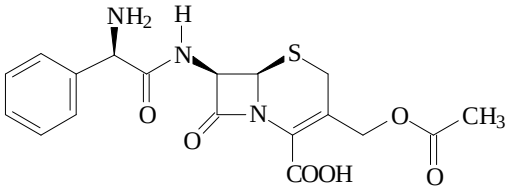
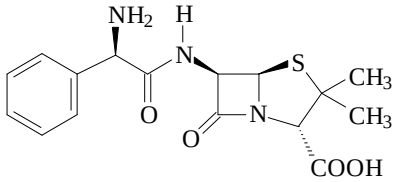
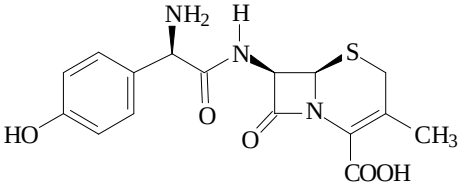
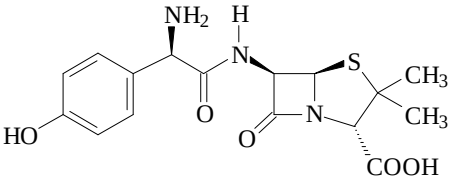
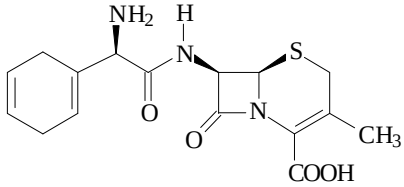
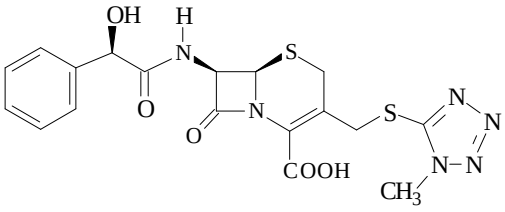
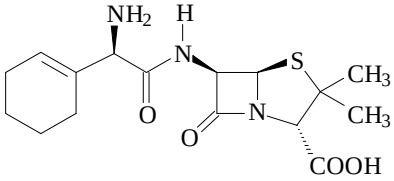
Abbreviations: E, enzyme; AEH, α-amino acid ester hydrolase; PA, penicillin G acylase; PVA, penicillin V acylase; GA, glutaryl acylase; CA, cephalosporin C acylase; Sub, subunit composition with their individual masses; Loc, localization; I, intracellular; P, periplasmic; E, extracellular; C, cytoplasmic; n.d. not determined. Gene, gene is cloned (+) or not cloned (-). Score gives the percent of identity with PA from *E. coli* determined with Clustal W. Symbols: •, not included in comparison study; and †, two cephalosporin acylases are found in this strain, a glutaryl acylase and a cephalosporin C acylase.

substrate for hydrolysis they are divided into five groups: penicillin G acylases (PAs), penicillin V acylases (PVAs), ampicillin acylases or more correctly α-amino acid ester hydrolases (AEHs), cephalosporin C acylases (CAs) and glutaryl acylases (GAs) (see Introduction for further information). Genetic and structural characterization of these β-lactam acylases showed that they all belong to the N-terminal nucleophile (Ntn) hydrolase superfamily (Oinonen and Rouvinen, 2000), except for the AEHs, of which up to recently (*Chapter 3* and further) no genetic or structural data was available.

The large scale chemical hydrolysis of fermentatively produced antibiotics is now efficiently replaced by an enzymatic cleavage process using β-lactam acylases such as penicillin G acylase from *Escherichia coli* and penicillin V acylases from *Bacillus sphaericus* (Bruggink, 2001; Shewale and Sudhakaran, 1997). In order to replace the subsequent chemical coupling steps that are applied in the production of semi-synthetic β-lactam antibiotics a suitable enzyme is needed. Several organisms have been described to produce enzymes that can perform this type of synthetic reaction. The first β-lactam antibiotic synthetic abilities were reported for penicillin acylase (PA) from *E. coli* in 1960 (Savidge, 1984; Vandamme and Voets, 1974). Coupling reactions performed with PA and free carboxylic acids as precursors of the side chains yielded only very low amount of antibiotics, since the thermodynamic equilibrium favors the hydrolysis products (Margolin *et al*., 1980). However, the use of activated acyl precursors (either as an ester or an amide) leads to a kinetically controlled process with a high level of transient product accumulation. It is desirable that at the point during the conversion where product levels are highest only a low amount of hydrolysis product is present. This hydrolysis product can be formed directly from the activated precursor and/or indirectly from the product. The synthetic performance of the conversion process depends both on reaction conditions and on the enzyme properties. Besides the PA from *E. coli* and the PAs from *Kluyvera citrophila* and *Bacillus megaterium* (Hernández-Justiz *et al*., 1999), the purified AEHs from *Acetobacter turbidans* ATCC 9325 (Ryu and Ryu, 1988; Takahashi *et al*., 1974), *Xanthomonas citri* IFO 3835 (Kato *et al*., 1980b), *Pseudomonas melanogenum* (Kim and Byun, 1990) were also found to be able to couple activated side chains to free β-lactam nuclei.

From 1972 up to now several organisms have been described to produce enzymes with AEH activity (Dharmarajan *et al*., 1994; Fujii *et al*., 1976; Takahashi *et al*., 1972) of which five were purified and studied more intensively (Table 1 and 3). The substrate range of the AEHs appears to be narrow, since the enzymes show a very high selectivity for an amino group on the C_α-position and prefer esters to amides (Kato *et al*., 1980b). However, if the acyl moiety of the substrate or acyl donor carries an α–amino group various β-lactam nuclei are accepted in hydrolysis and synthesis reactions, including 7- amino-desacetoxycephalosporanic acid (7-ADCA), 7-aminocephalosporanic acid (7-ACA), 6-APA, and 7-amino-3-((1H-1,2,3-triazol-4-ylthio)methyl)-cephalosporanic acid (7ATTC). In addition, side chains with rings that have a hydroxyl group on the *para* position or that are partially unsaturated are accepted both in hydrolysis and in transfer reactions (Table 2). Moreover, despite the high specificity for side chains with a phenylglycyl

Table 2. β-Lactam antibiotic substrate range of the AEHs.

β-Lactam antibiotic		Reference
Cephalexin		Kato *et al.*, 1980b; Nam *et al.*, 2001; Ryu and Ryu, 1988; Takahashi *et al.*, 1974
Cephaloglycin		Kato *et al.*, 1980b; Ryu and Ryu, 1988
Ampicillin		Kato *et al.*, 1980b; Ryu and Ryu, 1988; Takahashi *et al.*, 1974
Cephadroxil		Ryu and Ryu, 1988
Amoxicillin		Kato *et al.*, 1980a; Ryu and Ryu, 1988; Takahashi *et al.*, 1977
Cephradine		Fujii *et al.*, 1976
Cefamandole		Hernández-Jústiz *et al.*, 1999
Cyclacillin		Kato *et al.*, 1980b; Takahashi *et al.*, 1972

group, synthesis of cefamandole, which has a hydroxyl group on the α-position, has also been observed (Table 2). Additionally, hydrolysis and acyl transfer to 7-aminodesacetoxy-cephalosporanic acid (7-ADCA) has been found for the methyl and ethyl esters of all amino acids, except for those of asparagine, glutamine and aspartic acid, which were not tested (Kato *et al*., 1980b; Takahashi *et al*., 1974).

In contrast to a thermodynamic coupling, in which the enzyme only determines the time in which the reaction equilibrium is reached, in a kinetically controlled conversion the enzyme does influence the course of the reaction. Kinetic and structural studies have shown that PA from *E. coli* catalyzes hydrolysis and transfer reactions via an acyl-enzyme intermediate, as also occurs during the hydrolysis of peptides by chymotrypsin. The same was proposed for the AEHs of *A. turbidans, X. citri* and *Xanthomonas* sp. (Blinkovsky and Markaryan, 1993; Kato, 1980; Nam *et al*., 1985; Takahashi *et al*., 1974). According to the kinetic scheme for such a conversion, one of the factors that determine the maximum level of product accumulation is the factor α (Alkema, 2002; Gololobov *et al*., 1989), which is the ratio between the specificity (k_{cat} over K_M) for the antibiotic and the acyl donor. A relatively high specificity for the acyl donor corresponds to a small value for α, which is needed for a high yield. When we calculate the α-values for ampicillin and cephalexin of the different AEHs it appears that, unlike with *E. coli* PA, the specificity for the α-amino substituted acyl donor (ester) is higher than for the corresponding antibiotic (Table 3). The resulting lower α values suggest a high potential for the AEHs in the synthesis of these α-amino substituted β-lactam antibiotics.

Although several attempts have been undertaken, no gene encoding an AEH has been cloned since the discovery of the AEHs 30 years ago (Alonso and García, 1996; Nam and Ryu, 1988). Based on differences in size, pH-optimum and subunit composition (Table 1), it is expected that the AEHs differ significantly at the genetic and structural level from the other β-lactam acylases, which belong to the Ntn-hydrolase superfamily. The subunit size of all the known AEHs and the amino acid composition of the AEHs from *X. citri, A. turbidans* and *P. melanogenum* are quite similar, indicating that the various AEHs may belong to a related group of enzymes that forms a separate class of β-lactam acylases (Kim and Byun, 1990).

To judge the possible advantages of the AEHs in the synthesis of α-amino substituted β-lactam antibiotics we tested five AEHs and seven other enzymes with β-lactam acylase activity. For this, cell-free extracts (CFEs) of the different acylase-producing organisms were made and used to determine the acylase activity with chromogenic reference substrates. Of those CFEs with β-lactam acylases that accept an α-amino group, the ampicillin hydrolytic and synthetic activities were evaluated to select the best enzyme for ampicillin synthesis.

MATERIALS AND METHODS

Bacterial strains, growth conditions and extract preparation

The strains Acetobacter pasteurianus ATCC 9325 (called here A. turbidans), Acetobacter pasteurianus ATCC 6033, Kluyvera cryocrescens DSM 2660 (ATCC 21285), Pseudomonas diminuta N176, Bacillus sphaericus

Table 3. Kinetic parameters for AEHs and PA from *E. coli* (PA).

Organism	PGM			Cefalexine				Ampicillin			
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}.s^{-1}$)	α	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}.s^{-1}$)	α
X. citri IFO 3835	11 x 10^{3} [°]	8.3 [°] 11* 14.5 [#]	1.3 x 10^{3} [°]	3.2 x 10^{3} [°]	3.0 [°] 9.3*	1.1 x 10^{3} [°]	0.8				
Xanthomonas sp. [x]	1 x 10^{3}	3.6	277	71	0.35	203	0.8	17.5	0.6	29	0.1
A. turbidans ATCC 9325		4.9 [+] 4.3 [•] 4.0 [#]			1.5 [+] 3.7 [•] 3.0 [#]						
P. melanogenum IFO 12020 [§]					1.6				4.3		
E. coli ATCC 11105	50 [†]	12.5 [†]	4 [†]	57 [‡]	1.2 [‡]	48 [‡]	12	30 [†]	2.5 [†]	12 [†]	3

Symbols: [°], Kato *et al*., 1980b; *, Nam *et al*., 1985; [#], Rhee *et al*., 1980; [x], Blinkovsky and Markaryan, 1993'[+], Ryu and Ryu, 1988; [•], Takahashi *et al*., 1974;[#], Nam *et al*., 2001; [§], Kim and Byun, 1990; [†], Alkema *et al*., 2003; and [‡], Alkema *et al*., 1999. The α-values were calculated from the available parameters.

ATCC 14577, *Xanthomonas maltophilia* IFO 12020 (previously called *Pseudomonas melanogenum*) and *Xanthomonas citri* IFO 3835 were provided by DSM-Gist Brocades (Delft, The Netherlands). The strain *Arthrobacter viscosus* ATCC 15294 was retrieved from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). Cells of *Achromobacter* B-402-2 NRRL B-5393 were kindly provided by the National Center for Agricultural Utilization Research (Peoria, USA).

All strains were cultivated at 30 °C on the media indicated in the literature, except for *X. maltophilia*, which was grown on 20 g/l trypton and 10 g/l yeast and *P. diminuta*, which was grown on nutrient broth. Furthermore, *A. viscosus* was grown on corynebacterium medium and *Achromobacter* NRRL B-5393 was grown on Nutrient Broth, both as prescribed by the supplier. From 100 ml cultures of each organism the cells were harvested by centrifugation for 10 min at 6,000 g. The cells were washed with 10 ml and resuspended in 5 ml of 50 mM Na-phosphate buffer, pH 7.5. A clear cell-free extract (CFE) was made by sonification and subsequent centrifugation for 10 min at 16,000 g. From *B. megaterium* ATCC 14945, *E. coli* ATCC 11105 and *Pseudomonas sp.* FERM BP 817 (SE83) a crude enzyme preparation was kindly provided by DSM-Gist Brocades.

Materials

D-2-Nitro-5-[(phenylglycyl)amino]benzoic acid (NIPGB) was obtained from Syncom (Groningen, The Netherlands). 6-Amino-penicillanic acid (6-APA), D-phenylglycine methyl ester (PGM), D-phenylglycine amide (PGA), ampicillin and glutaryl-4-nitro anilide

(GNA) were provided by DSM Life Sciences (Delft, The Netherlands). D-Phenylglycine (PG) and 2-nitro-5-[(phenylacetyl)amino]benzoic acid (NIPAB) were obtained from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands).

Activity assays

Chromogenic activity assays - The chromogenic substrates NIPGB and NIPAB were used to measure the activity of the β-lactam acylases (Alkema et al., 1999). For this, each extract was incubated overnight at room temperature in a microtiter plate with 2.4 mM NIPGB in 50 mM phosphate buffer (pH 6.2) or 150 µM NIPAB in 42 mM Na-phosphate buffer (pH 7.2). Additionally, the cephalosporin acylases (CA) were incubated with 10 mM GNA in 10 mM Tris.HCl (pH 7.2). Because of the turbidity, the activity of each extract was judged either positive or negative against the controls (only substrate and only CFE) by eye. The conversion of NIPGB was also tested using so-called NIPGB-paper, as described by Zhang et al. (1991) for NIPAB-paper. A yellow color appearing around a colony during incubation of NIPGB-paper at room temperature indicated the possible presence of an α-amino acid ester hydrolase. In both methods, the colonies were tested with and without permeabilization. Permeabilization in microtiter plates was achieved by adding chloroform to the cell suspension to a final concentration of 5% (v/v). Permeabilization for the NIPGB-paper method was achieved by exposing the NIPGB-paper with colony material on it to saturated chloroform vapors for 15 min at room temperature.

HPLC-based activity assays - The synthesis and hydrolysis of ampicillin was performed at 4 °C and the concentrations of both ampicillin and the hydrolysis product phenylglycine (PG) were determined by HPLC using a Chrompak C18 column connected to Jasco PU-980 pumps and a Jasco MD-910 detector set a 214 nm. PG, 6-APA and PGA were eluted isocratically in 50 mM Na-phosphate pH 3, PGM and ampicillin were subsequently eluted in a binary gradient from 50 mM Na-phosphate pH 3 to 15% acetonitrile, 25 mM Na-phosphate. For ampicillin synthesis 200 µl of the CFEs and 10 µl of the *E. coli* extract were incubated with 20 mM 6-APA and 20 mM activated side chain (PGA or PGM) in a total volume of 1.5 ml for 60 min. The reaction was performed at four different pH-values, pH 7.2 (83 mM Na-phosphate), pH 6.2 (idem), pH 5.2 (83 mM Na-acetate) and pH 4.2 (83 mM Na-formate). For hydrolysis reactions the extracts containing the *E. coli* PA and those with AEHs were incubated with 4 mM and 3 mM ampicillin, respectively, for 60 min at pH 6.2 (83 mM Na-phosphate). Samples were taken from the incubations and quenched with one sample volume of 1 N HCl and immediately returned to the starting pH value with a predetermined amount of 2 N NaOH. For analysis, a 10-fold dilution of the sample was made with 200 mM Na-phosphate, pH 3.0, and the diluted sample was injected into the HPLC. For the initial synthesis rate, the slope from the synthesis curve from 0 to 5 min was determined. Additionally, after 60 min the amounts of synthesized and hydrolyzed ampicillin per mg of protein were determined. Dividing the amount of synthesized ampicillin per mg by the amount ampicillin hydrolyzed per mg of protein gives a synthesis over hydrolysis ratio at 60 min, or S/H ratio.

For the synthesis of cephalexin, the suspended cells or enzyme solutions were incubated with 30 mM 7-ADCA and 15 mM D-

PGM in 50 mM sodium-phosphate buffer, pH 6.2, at 30 °C. Before analysis the samples were quenched and diluted 50-fold by the addition of HPLC eluent (50 mM Na-phosphate, pH 3). The synthesis and hydrolysis reactions were followed by reverse-phase HPLC as described for ampicillin detection (Alkema *et al.*, 2000). One unit of activity (U) is defined as the amount of enzyme that is needed to produce one µmol of product per min under the conditions used.

Purification of *A. pasteurianus* AEH activity

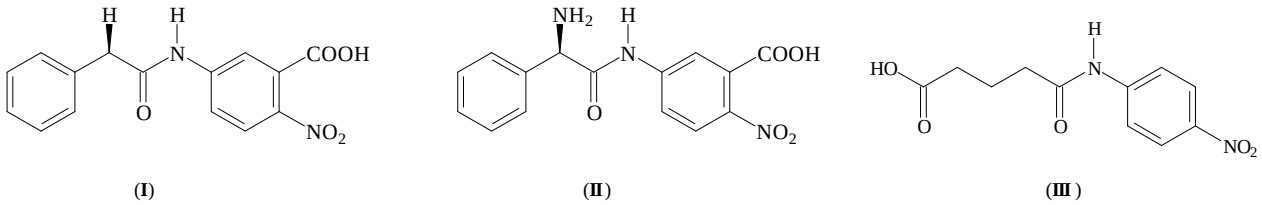
For the purification of the *A. pasteurianus* AEH activity 1.2 l cell-free extract (CFE, 0.7 g of protein, provided by DSM-Gist) was brought to 50 mM Tris-HCl (pH 7.5) containing 1 mM MgCl and was subsequently incubated with approximately 3 to 5 mg DNAse and RNAse (both from Roche Diagnostics GmbH, Basel, Switzerland) for 12 h at 4 °C. All subsequent steps were performed at 4 °C. During the purification the AEH activity was followed with NIPGB in microtiter plates and cephalexin synthesis was measured as described above. After centrifugation (2 h at 18,000 g) the extract was applied on a Q-sepharose fast-flow column (5- by 15-cm, Amersham Pharmacia Biotech Ltd., Hertfordshire, United Kingdom) which was equilibrated with 50 mM Tris-HCl (pH 7.5). The AEH activity eluted in the unbound fraction and was subsequently applied on a Resource phenyl column (2.6- by 4-cm column, Amersham Pharmacia) equilibrated with 40% ammonium sulfate. The AEH activity eluted from the column at 12% to 15% ammonium sulfate and was purified further on a Sephacryl S300 column (1.6- by 55-cm column, Amersham Pharmacia) equilibrated with 50 mM Tris, pH 7.5, 0.15 M NaCl. Subsequently the AEH activity was fractionated on a Resource phenyl column (prepacked, 0.6- by 3 cm, Amersham Pharmacia). The $(NH_4)_2SO_4$ concentration was lowered stepwise from 40 to 20% in 15 and from 20 to 0% in 30 column volumes. The active fractions eluted at 16-11% ammonium sulfate and were collected and brought on a Superdex 200 column (prepacked, 0.5- by 30-cm column, Amersham Pharmacia). This column was equilibrated and run with 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl. Active protein was pooled and analyzed by SDS-PAGE and native-PAGE to identify proteins with AEH activity.

RESULTS AND DISCUSSION

NIPGB hydrolysis activity of acylase producing strains

In search for enzymes with attractive properties for the enzymatic synthesis of α-amino substituted semi-synthetic β-lactam antibiotics, we characterized the catalytic behavior of a range of microbial acylases that are active in the hydrolysis and/or synthesis of β-lactam antibiotics. Of the cloned β-lactam acylases, the comparison included four penicillin G acylases, one glutaryl acylase, one cephalosporin acylase and one penicillin V acylase (Table 1). Of the less well-studied α-amino acid ester hydrolases (AEHs), five were included (Table 1).

The cell-free extracts of the organisms were tested with the standard PA chromogenic substrate NIPAB (Fig. 1, **I**), with NIPGB (Fig. 1, **II**), which is the α-amino substituted variant of NIPAB, and with glutaryl *p*-NA (Fig. 1, **III**) to confirm the activity and the substrate specificity of the expressed acylase. NIPGB in particular was used to test the ability of the acylases to accept an α-amino acid group.



(**I**) (**II**) (**III**)

Figure 1. Chromogenic substrates. (I) NIPAB; (II) NIPGB and (III) glutaryl-pNA.

For each extract acylase activity was found, except for the extract from *B. sphaericus* (Table 4). This extract showed no activity with either of the chromogenic substrates, although activity with NIPAB was expected since hydrolysis of penicillin G has been reported for the penicillin V acylase of *B. sphaericus* (Pundle and SivaRaman, 1997). Apparently, the used strain was not correct or did not express an active β-lactam acylase and it was therefore excluded from further testing. All the active PA and AEH containing extracts showed hydrolytic activity with NIPGB (Table 4). The AEH containing extracts only hydrolyzed NIPGB and not NIPAB, which is in agreement with the reported α-amino specificity of AEHs (Fujii *et al*., 1976; Kato *et al*., 1980b; Kim and Byun, 1990; Margolin *et al*., 1980; Takahashi *et al*., 1974; Takahashi *et al*., 1972). The extracts from *Pseudomonas* sp. SE83 and from *P. diminuta* N176, containing a glutaryl 7-ACA acylase and cephalosporin acylase, respectively, did not hydrolyze NIPAB or NIPGB. Obviously, the substrate specificities of the acylases produced by these organisms is limited to β-lactam antibiotics with linear side chains or side chains with charged groups (acid or amine groups) and does not include side chains that consist of a hydrophobic phenyl moiety (Fritz-Wolf *et al*., 2002).

Table 4. The activity of the selected organisms on chromogenic substrates and in the synthesis of ampicillin.

Organism	Enzyme	*GNA*	NIPAB	NIPGB	Ampicillin
X. citri IFO 3835	AEH	n.d.	-	+	+
A. turbidans ATCC 9325	AEH	n.d.	-	+	+
X. maltophilia IFO 12020	AEH	n.d.	-	+	+
E. coli ATCC 11105	PA	n.d.	+	+	+
K. cryocrescens ATCC 21285	PA	n.d.	+	+	n.d.
B. megaterium ATCC 14945	PA	n.d.	+	+	+
A. viscosus ATCC 15294	PA	n.d.	+	+	n.d.
Pseudomonas sp. SE83	GA	+	-	-	n.d.
P. diminuta N176	CA	+	-	-	n.d.
B. sphaericus ATCC 14577	PVA	n.d.	-	-	n.d.
A. pasteurianus ATCC 6033	AEH	n.d.	-	+	+
Achromobacter NRRL B-5393	AEH	n.d.	-	+	+

Symbols: -, not active; +, active; n.d., not determined.

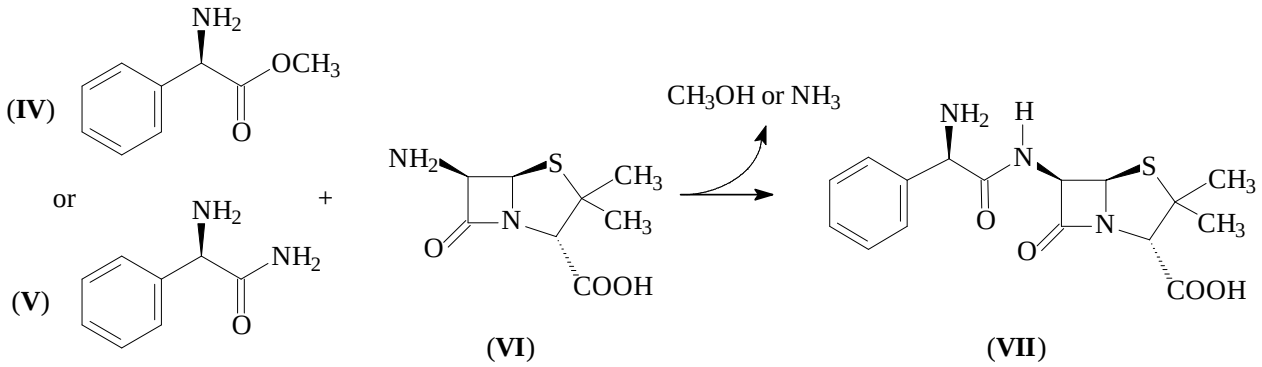


Figure 2. **Synthesis reaction of ampicillin catalyzed by β-lactam acylases.** Ampicillin (**VII**) can be synthesized from PGM (**IV**) or PGA (**V**) and 6-APA (**VI**).

Ampicillin synthesis

To see if the enzymes that accept an α-amino group in a hydrolysis substrate can also synthesize α-amino substituted β-lactam antibiotics, the NIPGB active extracts containing the AEHs, the periplasmic PA of *E. coli*, and the extracellular PA of *B. megaterium* were subsequently tested for their ability to synthesize ampicillin (Fig. 2, **VII**) from the activated side chain donor PGM (Fig. 2, **IV**) or PGA (Fig. 2, **V**) and the free nucleus 6-APA (Fig. 2, **VI**). The use of CFEs in these synthesis experiments might give misleading results if other esterases or amidases are present that influence the course of the synthesis reactions. Such contaminating enzymes could potentially hydrolyze the activated precursor or degrade the product, both resulting in a lower level of product accumulation. Therefore, next to the product concentration after 60 min, we also determined the initial rate of synthesis with the assumption that the influence of contaminating enzymes and products (product inhibition) is negligible in the beginning of the synthesis reaction.

Incubation of the extracts with the free 6-APA nucleus and the activated phenylglycine side chain (PGA or PGM) showed that all the enzymes that earlier were able to hydrolyze NIPGB were also able to synthesize ampicillin (Fig. 3A). The enzymes from *E. coli* and *B. megaterium* were able to use both side chain donors while the AEHs showed transfer of the side chain to 6-APA only when PGM was used (only tested at pH 7.2, data not shown). There was no detectable formation of PG from PGA by the AEHs, which is in agreement with the low activity of AEHs for amides reported in literature (Kato *et al*., 1980b). The *E. coli* enzyme performed the best ampicillin synthesis (highest ampicillin production in combination with low hydrolysis product formation) when using PGA and the *B. megaterium* enzyme when using PGM as the side chain donor. Therefore the data obtained with these side chain donors were shown in the comparison with the other enzymes (Fig. 3). The ampicillin synthesis experiments clearly showed that the AEHs from *A. turbidans*, *X. citri* and *A. pasteurianus* and the PA from *E. coli* were the only enzymes that had both high initial synthesis rates and high levels of product accumulation (Table 5 and Fig. 3). Within the applied incubation period, a maximum in the product

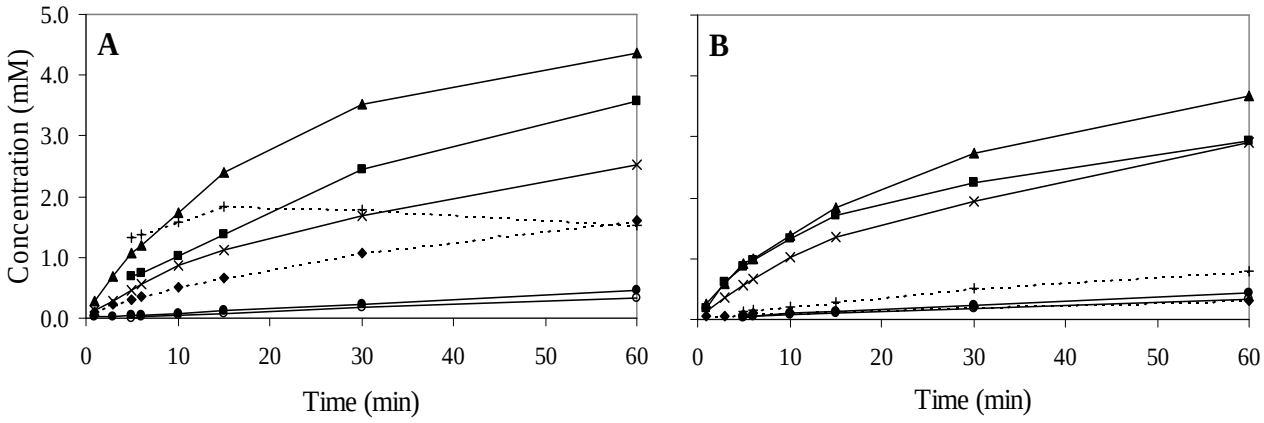


Figure 3. Ampicillin synthesis at pH 7.2 (A) and pH 6.2 (B) of the AEHs (—) and PAs (---). Cell-free extracts were incubated with 20 mM 6-APA and 20 mM PGM (20 mM PGA for *E. coli*) at 4°C in a total volume of 1.5 ml. The amount of protein added is indicated behind the different organisms. Symbols: (▲), *A. turbidans*, 1.26 mg of protein extract was added; (■), *A. pasteurianus*, 0.48 mg; (×), *X. citri*, 2.6 mg; (♦), *E. coli*, 0.4 mg (0.7 NIPAB units); (+), *B. megaterium*, 3 mg; (•), *X. maltophilia*, 2 mg; (o), *Achromobacter*, 0.6 mg.

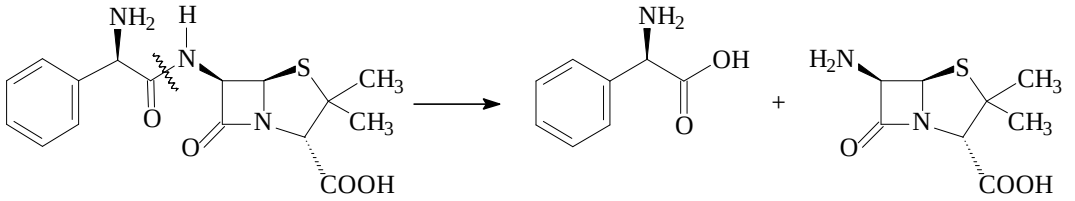
accumulation curve (Q_{max}) was only reached in the synthesis reaction catalyzed by the PA of *B. megaterium* at pH 7.2 (Fig. 3A). However, since the Q_{max} is not dependent on the enzyme concentration (Gololobov *et al*., 1989; Kasche, 1986) it is clear that the AEHs from *A. turbidans*, *X. citri* and *A. pasteurianus* and maybe the PA from *E. coli* can reach higher Q_{max} values than PA of *B. megaterium*.

Synthesis at lower pH is beneficial for the stability of 6-APA. Therefore, the enzymes were tested for their ability to synthesize ampicillin at two pH-values, pH 6.2 and 7.2, i.e. either close to the optimal pH-values of the AEHs or the PAs (Table 1). The AEHs were the only enzymes which synthesized similar amounts of ampicillin at pH 6.2 (Fig. 3B) compared to pH 7.2. The AEHs from *X. maltophilia* and *Achromobacter* are probably poorly expressed or have low catalytic activity since they synthesized only very small amounts of ampicillin at both pH-values. To see if the AEHs are able to synthesize at even lower pH-values, the AEHs from *X. citri* and from *A. pasteurianus* were tested at pH 5.2 and 4.2. The AEH from *X. citri* showed the highest synthetic activity at pH 6.2, which was reduced to 40% at pH 5.2 and to 12% at pH 4.2. The synthesis activity with the *A. pasteurianus* extract decreased much faster with decreasing pH value, i.e. from 100% at pH 7.2 to 8% at pH 5.2 and to 1% at pH 4.2. Therefore, the *X. citri* AEH is considered the best enzyme for reactions at lower pH-values.

In conclusion, crude extracts of the AEHs from *X. citri*, *A. turbidans* and *A. pasteurianus* synthesized ampicillin at higher initial rates, produced more ampicillin and had a higher ampicillin synthesis activity at low pH-values than the penicillin acylases.

Ampicillin hydrolysis versus synthesis

In addition to high synthesis activity, a low ampicillin hydrolysis activity would be beneficial for high product accumulation. Therefore, the hydrolytic activity was determined for those enzymes that showed good synthesis activity of ampicillin at pH 6.2. *X. citri*, *A. turbidans* and



(**VII**) (**VIII**) (**VI**)

Figure 4. Hydrolysis of ampicillin (VII) by β-lactam acylases. Hydrolysis of the amide bond results in PG (**VIII**) en 6-APA (**VI**). The waved line indicates the amide bond hydrolyzed by β-lactam acylases.

A. pasteurianus, and for comparison PA of *E. coli*, was included. Due to variations in the level of expression, the relative amount of active enzyme may vary in the extracts of the different organisms. Therefore, to obtain a meaningful value for each enzyme, the ratio of the specific synthesis and hydrolysis activities (S/H ratio) was determined.

The extracts from the selected organisms were incubated with ampicillin at pH 6.2 and the hydrolysis was measured by the rise of PG to correct for other ampicillin degrading enzymes (Fig. 4, (**VIII**)). The results showed that the PA containing extract from *E. coli* hydrolyzed the most ampicillin per mg of protein, followed by the AEH containing extracts from *X. citri* and the *Acetobacter* strains (Table 5). Calculations of the S/H ratios showed that the AEHs had higher ratios than *E. coli* PA indicating that the AEHs have a lower affinity for the product (amide). This is in agreement with the identification of the AEHs as esterases, in contrast to *E. coli* PA, which is an amidase (Margolin *et al.*, 1980). For the extract of *A. pasteurianus* and *A. turbidans* the lowest ampicillin hydrolysis per mg of protein was found (Table 5). The highest S/H ratio was found for *A. pasteurianus.*

In conclusion, in CFEs the AEHs expressed by *A. turbidans*, *A. pasteurianus* and *X. citri* have higher initial specific synthesis rates than partially purified PA from *E. coli* and additionally showed high synthesis over

Table 5. Ampicillin synthesis and hydrolysis at pH 6.2.

Organism	Acyl donor	Initial synthesis rate (mU/mg)	Synthesized ampicillin (µmol/mg)	Hydrolyzed ampicillin (µmol/mg)	S/H ratio
A. pasteurianus	PGM	251	4.4	0.4	10
A. turbidans	PGM	97	2.2	0.4	5.5
X. citri	PGM	62	1.7	0.7	2.4
E. coli	PGA	38	1.1	1.2	0.9

The amount of ampicillin synthesized or hydrolyzed per mg of protein was determined after 60 min as explained under Material and Methods.

hydrolysis ratios. Thus, the AEH from *A pasteurianus* seems the most interesting candidate for further investigation. However, a more conclusive evaluation of these AEHs requires comparison with pure enzymes. To provide enough material for these studies, overexpression systems are needed and therefore it was decided to clone the genes encoding the individual AEHs starting with the AEH from *A. pasteurianus*.

Attempts to clone the gene encoding AEH from *A. pasteurianus*

A cosmid library of total *A. pasteurianus* DNA was made in *E. coli* HB101 as described in *Chapter 3*. The resulting library consisted of 690 colonies with insert sizes of 22 kb and higher, corresponding to 97% completeness.

It was attempted to identify a clone containing the AEH gene by screening the library for conversion of the chromogenic substrate NIPGB using microtiter plates. Since the AEH from *A. pasteurianus* could be located intracellularly, the colonies were screened with and without permeabilization. No positive clones were found, neither in microtiter plates, nor when the colonies were transferred to and tested with NIPGB-paper. Additionally, the library was screened for auxotrophic complementation of the leucine deficiency of the library host with D-phenylglycine-L-leucine as the sole source of leucine on minimal medium plates, but again no positive clone was found. Finally, the clones were tested for their ability to synthesize cephalexin. Control tests with 49 non-acylase producing *E. coli* HB101 clones and one *A. pasteurianus* colony indicated that one cephalexin producing clone could be detected among 49 negative clones by HPLC measurements. Therefore, sets of 50 clones of the library were pooled and incubated with PGM and 7-ADCA and the reaction was followed by HPLC. Although this was considered the most sensitive screening method, no cephalexin-producing clones were found.

The absence of a positive clone in the library might be due to i) lack of expression of AEH in the *E. coli* host from the *A. pasteurianus* promoter present in the low copy number cosmid (pLAFR3), ii) poor post-translational activity in the host, or iii) an incomplete library. The expression and/or processing of the enzyme might be better in a different host strain. Therefore, the whole library was conjugated to *Pseudomonas* US2 as described by Janssen *et al.* (1989) with an efficiency of 90%. Unfortunately, neither hydrolysis of NIPGB nor synthesis of cephalexin was detected in this host either.

To make it possible to design a genetic probe for the screening of the library we attempted to purify the AEH from *A. pasteurianus*. The AEH activity could be purified 71 times, however, due to the many impurities co-eluting with the AEH activity the yield was very low (Table 6) and no pure protein could be obtained. On the gel filtration columns the native AEH activity eluted at 150 kDa. SDS-PAGE analysis of the purification showed that the increase in activity coincided with the increase in concentration of a protein with a subunit size of 52 kDa, which differs significantly from the subunit sizes of 70 and 72 kDa found for *A. turbidans* and *X. citri* (Kato *et al*., 1980a; Takahashi *et al*., 1974). After isolation from an SDS-PAGE gel, the following N-terminal amino acid sequence was found for the 52 kDa protein: Met-Arg-Gln-Asp-Phe-Ile-Ser-Thr-Gln-Leu-Leu-Val-Ala (90% certainty). Database searches (advanced BLAST and PSI-BLAST (Altschul *et al*., 1997)) revealed no homology with a known protein nor does this

Table 6. Purification of the AEH from *A. pasteurianus* ATCC 6033.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (cexU)	Specific activity (cexU/mg)	Purification (fold)	Recovery (%)
CFE	1167	665	499	0.7	1	100
Q-sepharose	1101	359	480	1.3	1.9	96
Resource phenyl	49	25	200	8.0	11	40
Sephacryl S300	4.8	0.74	10	14	20	2
Resource phenyl & Superdex 200	0.25	0.06	3	50	71	0.6

sequence show any homology with one of the recently cloned AEHs (*Chapter 3* and further). Attempts to amplify and isolate the complete gene for this protein using degenerate primes based on this sequence with the LA PCR *in vitro* cloning kit from Takara (see *Chapter 3*) did not lead to more nucleotide sequence information.

The cloning of the *A. pasteurianus* AEH-encoding gene was finally accomplished after the sequence of the corresponding gene from *A. turbidans* (*Chapter 3*) was determined. In retrospect, three copies of the gene were present in the *A. pasteurianus* genomic cosmid library, indicating that indeed lack of expression or processing prevented the location of the gene by activity assays. The sequence of the AEH gene from *A. pasteurianus* will be discussed in more detail in the Summary and Concluding Remarks (*Chapter 7*) of this thesis.

CONCLUSIONS

Measurements with cell-free extracts showed that the α-amino acid ester hydrolases (AEHs) present in *A. pasteurianus*, *X. citri* and *A. turbidans* are potentially attractive for the synthesis of semi-synthetic antibiotics that contain an α-amino group on the phenylacetyl side chain. Although they do convert the coupling product ampicillin (amide), their selectivity for the acyl donor (ester) is promising. The best ampicillin synthesizing properties were found for the AEH expressed by *A. pasteurianus*. To be able to compare and study the AEHs in more detail, pure enzyme is needed and thus the first attempts to clone the gene encoding the AEH from *A. pasteurianus* were undertaken. Unfortunately, no expression of this AEH was detected in a gene library of *A. pasteurianus* in *E. coli*. Additionally, the amount of AEH in the wild-type organism was too low to obtain a sufficient amount of enzyme for identification or for designing a probe. Therefore, further attempts to clone an AEH gene were focussed on *A. turbidans*. The remaining chapters of this thesis will describe the cloning of AEH-encoding genes of *A. turbidans* and *X. citri* as well as the kinetic and structural characterization of the corresponding proteins.

Acknowledgements

We thank Piet Wietzes for his technical assistance

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Cloning, sequence analysis, and expression in *Escherichia coli* of the gene encoding an α-amino acid ester hydrolase from *Acetobacter turbidans*

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ABSTRACT

The α-amino acid ester hydrolase from *Acetobacter turbidans* ATCC 9325 is capable of hydrolyzing and synthesizing β-lactam antibiotics, such as cephalexin and ampicillin. N-terminal amino acid sequencing of the purified α-amino acid ester hydrolase allowed cloning and genetic characterization of the corresponding gene from an *A. turbidans* genomic library. The gene, designated *aehA*, encodes a polypeptide with a molecular weight of 72,000. Comparison of the determined N-terminal sequence and the deduced amino acid sequence indicated the presence of an N-terminal leader sequence of 40 amino acids. The *aehA* gene was subcloned in the pET9 expression plasmid and expressed in *Escherichia coli*. The recombinant protein was purified and found to be dimeric with subunits of 70 kDa. A sequence similarity search revealed 26% identity with a glutaryl 7-ACA acylase precursor from *Bacillus laterosporus*, but no homology was found with other known penicillin or cephalosporin acylases. There was some similarity to serine proteases, including the conservation of the active site motif, GxSYxG. Together with database searches, this suggested that the α-amino acid ester hydrolase is a β-lactam antibiotic acylase that belongs to a class of hydrolases that is different from the Ntn-hydrolase superfamily to which the well-characterized penicillin acylase from *E. coli* belongs. The α-amino acid ester hydrolase of *A. turbidans* represents a subclass of this new class of β-lactam antibiotic acylases.

INTRODUCTION

In search for microorganisms applicable in the biocatalytic production of semisynthetic antibiotics, *Acetobacter turbidans* ATCC 9325 was first described in 1972 by Takahashi *et al.* (Takahashi *et al.*, 1972) as an organism able to synthesize cephalosporins. Since only α-amino acid derivatives could act as substrates and due to the preference for esters over amides, the enzyme involved was named α-amino acid ester hydrolase (AEH) (Takahashi *et al.*, 1974).

A similar AEH (E.C. 3.1.1.43) activity has been described for several other organisms. These enzymes catalyze the transfer of the acyl group from α-amino acid esters to amine nucleophiles such as 7-aminocephem and 6-penam compounds (synthesis) or to water (hydrolysis) (Fig. 1). Presumably, an acyl-enzyme intermediate is involved in this transfer reaction (Blinkovsky and Markaryan, 1993; Takahashi *et al.*, 1974). These AEHs show promising properties for the industrial enzymatic production of semi-synthetic β-lactam antibiotics. Due to the preference for esters, it is conceivable that higher product (amide) accumulation can be reached in synthesis reactions using these enzymes than with the *Escherichia coli* penicillin G acylase (E.C. 3.5.1.11) (Hernández-Jústiz *et al.*, 1999; Ryu and Ryu, 1988; Takahashi *et al.*, 1974). Moreover, the enzyme of *A. turbidans* showed high selectivity toward the D-form of phenylglycine methyl ester (D-PGM, the activated acyl donor). This enables an ampicillin synthesis starting from a racemic mixture of acyl donors, which is not feasible with the *E. coli* penicillin acylase (Fernández-Lafuente *et al.*, 2001). In contrast to penicillin acylase from *E. coli*, it was claimed that the α-amino acid ester hydrolases are able to accept charged substrates (Blinkovsky and Markaryan, 1993). The lower pH

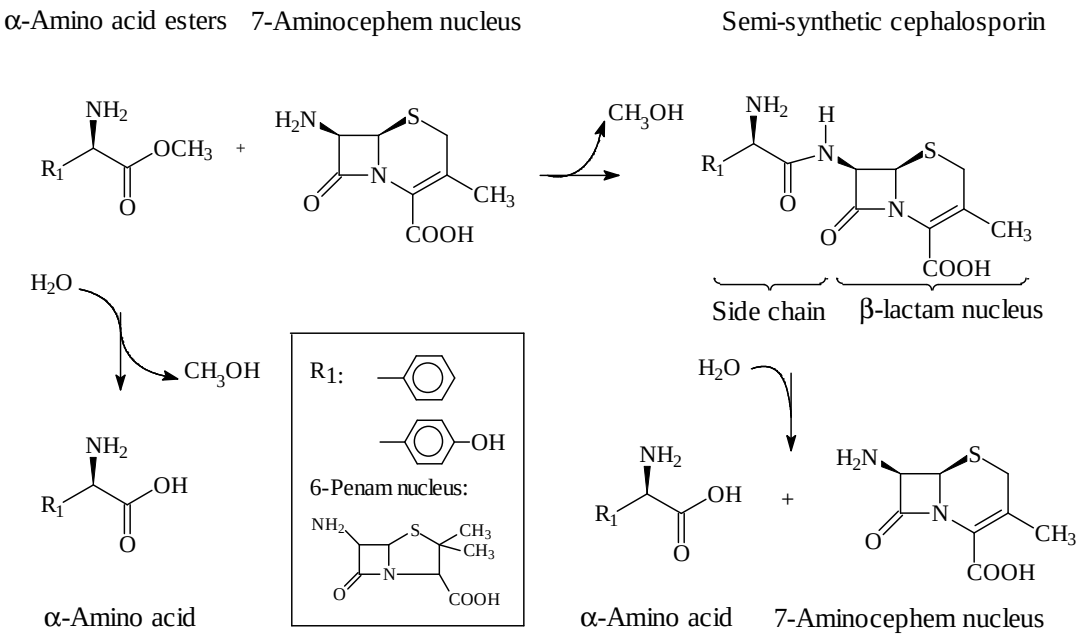


Figure 1. Example of synthesis and hydrolysis of β-lactam antibiotics catalyzed by α-amino acid ester hydrolase of *A. turbidans*. Shown as a 7-amincephem nucleus is 7-amino-desacetoxy-cephalosporanic acid, and 6-aminopenicillanic acid is depicted as a 6-penam nucleus.

optimum of the α-amino acid ester hydrolases, i.e. pH 6 compared to pH 7.5-8 for penicillin G acylases (Kutzbach and Rauenbusch, 1974), and the lack of inhibition by phenylacetic acid (Blinkovsky and Markaryan, 1993), a side product from the hydrolysis of penicillin G, are also interesting properties for biocatalytic applications.

The structural characterization of the AEHs is limited to the determination of the subunit size and the quaternary structure. The AEHs from *A. turbidans* ATCC 9325 (Ryu and Ryu, 1987; Takahashi *et al*., 1974), *Xanthomonas citri* IFO 3835 (Kato *et al*., 1980a) and *Pseudomonas melanogenum* IFO 12020 (Kim and Byun, 1990) have been purified. All three enzymes have similar subunit sizes of either 70 kDa, 72 kDa or both. However, there is some dissimilarity in the native subunit composition which was reported to be $\alpha_2\beta_2$ (Ryu and Ryu, 1987) for *A. turbidans*, α_4 for *X. citri* (Kato *et al*., 1980a) and α_2 for *P. melanogenum* (Kim and Byun, 1990). Cloning of the gene encoding AEH and using it for overproduction would be worthwhile since expression in the natural hosts is low, varying from 0.1 to 2% of the total cellular protein (Kato *et al*., 1980a; Kim and Byun, 1990, this study; Ryu and Ryu, 1987; Takahashi *et al*., 1974). In the past, effort has been put into cloning an *aehA* gene but this was unsuccessful (Alonso and García, 1996; Nam and Ryu, 1988).

In this paper we describe the cloning and genetic characterization of the α-amino acid ester hydrolase of *A. turbidans* ATCC 9325. We succeeded in producing active AEH in *E. coli* and report some kinetic and structural properties of the purified recombinant protein. The sequence analysis showed that the α-amino acid ester hydrolase is a member of a new class of β-lactam antibiotic acylases.

MATERIALS AND METHODS

Materials

D-2-Nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) was obtained from Syncom (Groningen, The Netherlands). 6-Aminopenicillanic acid (6-APA), 7-aminodesacetoxycephalosporanic acid (7-ADCA), D-phenylglycine methyl ester (D-PGM), D-phenylglycine amide (D-PGA), D-4-hydroxyphenylglycine methyl ester, amoxicillin, cefadroxil, glutaryl 7-aminocephalosporanic acid (glutaryl 7-ACA), adipoyl 7-ADCA and cephalexin were provided by DSM Life Sciences (Delft, The Netherlands). The oligonucleotides for cloning of the *aehA* gene were provided by Eurosequence BV (Groningen, The Netherlands).

Bacterial strains, plasmids and growth conditions

A. turbidans ATCC 9325 was grown at 30 °C in a 10 l fermentor on the medium described by Takahashi *et al.* (Takahashi *et al.*, 1974) without the addition of antifoam. Bacto-Peptone was purchased from Difco (Sparks, USA) and the meat extract was obtained from Fluka (Buchs, Switzerland). *E. coli* strains were grown in shake flasks at 30 °C on LB medium. Antibiotics were added to the media at the following final concentrations: tetracycline (Tc), 12.5 µg/ml; kanamycin (Km), 50 µg/ml; chloramphenicol (Cm), 34 µg/ml and ampicillin (Ap), 50 µg/ml. When required, isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added to a final concentration of 0.4 - 1 mM. *E. coli* HB101 (Boyer and Roulland-Dussiox, 1969) was used for cloning derivatives of pLAFR3 (Staskawicz *et al.*, 1987) and pEC (DSM life science, Delft, The Netherlands). *E. coli* strains BL21(DE3)pLysS (Promega) and TOP10F' (Invitrogen, Leek, The Netherlands) were used for cloning derivatives of pET9 (Promega Corporation, Madison, USA) and pCR-TOPO (Invitrogen), respectively.

DNA manipulation and sequencing

All chemicals used in DNA manipulation procedures were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and used as recommended by the manufacturer. The DNA sequences were determined at the Department of Medical Biology of the University of Groningen.

Isolation of α-amino acid ester hydrolase from *A. turbidans*

Cells of *A. turbidans* were harvested in the stationary phase by continuous centrifugation at 6,000 x *g*, washed twice with 10 mM potassium phosphate buffer (pH 6.2) and resuspended in this buffer. All further steps were carried out at 4 °C. A cell extract was made by sonification and cell debris was removed by centrifugation at 13,000 x *g* for 40 min. To the supernatant DNase and RNase (final concentration 6 mg/l each) were added in the presence of 5 mM $MgSO_4$. The solution was incubated for 3 h under mild stirring and centrifuged at 50,000 rpm in a type 50 Ti rotor (Beckman) for 60 min and then applied to a CM sepharose fast flow column (5 by 15 cm column, Amersham Pharmacia Biotech Ltd., Hertfordshire, United Kingdom) pre-equilibrated with 10 mM K_2HPO_4-KH_2PO_4 , pH 6.2. Prior to elution the non-binding proteins were washed from the column with equilibration buffer. The retained protein eluted in a linear gradient of 0-1 M KCl (30 ml/min) at 0.2 M. Activity containing fractions were pooled and $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M, after which the

pool was loaded on a hydrophobic interaction column (Resource Phenyl, 2.6 by 7.5 cm, Amersham Pharmacia) pre-equilibrated with 1.5 M $(NH_4)_2SO_4$, 50 mM Na-phosphate buffer, pH 6.2. After washing with the equilibration buffer the AEH eluted at 0.8-0.68 M $(NH_4)_2SO_4$ in a decreasing linear gradient from 1.5 M to 0 M $(NH_4)_2SO_4$ in 50 mM Na-phosphate buffer (pH 6.2) at 5 ml/min. Fractions that contained AEH were pooled and concentrated by ultrafiltration (YM30, Amicon bioseparations, Millipore, Bedford, USA) and loaded on a Superdex 200 HR 10/30 column (24-ml bed volume, Amersham Pharmacia). AEH was eluted at 1 ml/min in 50 mM Na-phosphate buffer (pH 6.2), 0.15 M NaCl.

Isolation of recombinant α-amino acid ester hydrolase from *E. coli*

The recombinant AEH was purified from *E. coli* BL21(DE3)pLysS (Cm^R) cells carrying the pETAT (Km^R) construct. The cells were harvested from two 2.5-l cultures by centrifugation and the crude extract was prepared as described above. The extract was loaded on a DEAE Sepharose column (5 by 13-cm column, Amersham Pharmacia) pre-equilibrated with 50 mM Na-phosphate buffer, pH 6.2. The AEH activity was eluted from the column in the non-binding fraction in the equilibration buffer at 30 ml/min. The activity was then applied to a CM-HAP (ceramic hydroxy apetite column, 2.6 by 11-cm column, Amersham Pharmacia) which was equilibrated with 50 mM Na-phosphate, pH 6.2. After washing with the equilibration buffer the AEH activity was eluted from the column at 275 mM Na-phosphate in a linear gradient of 50 to 500 mM Na-phosphate (pH 6.2) at 10 ml/min. The AEH was purified further to SDS-PAGE homogeneity by hydrophobic interaction and gelfiltration chromatography as described above.

Preparation and screening of the *A. turbidans* genomic library

Genomic DNA was isolated as described by Poelarends *et al.* (Poelarends *et al.*, 1998). An incubation of 30 min at 37 °C with proteinase K (0.10 mg/ml) after the first hour of incubation with SDS was added to the procedure. DNA of the cosmid pLAFR3 used for the construction of the gene library was isolated from *E. coli* HB101 according to the alkaline lysis method and purified by ultracentrifugation using a CsCl gradient (Sambrook *et al.*, 1989). The chromosomal DNA of *A. turbidans* was partially digested with *Sau*3A to yield fragments with an average size of 30-50 kb. These fragments were ligated in the cosmid pLAFR3 (Tc^r) which had been completely digested with *Bam*HI and dephosphorylated with alkaline phosphatase. *In vitro* packaging and infection of *E. coli* HB101 was carried out according to the recommendations of the manufacturer (Roche). Recombinant clones were stored at -80 °C in microtiter plates.

Colony hybridization was essentially carried out as described by Van Hylckama Vlieg *et al.* (Van Hylckama Vlieg *et al.*, 2000) using an AEH specific probe. An incubation of the membrane with proteinase K for 30 min at room temperature after fixation of the DNA was included in the procedure. After hybridization at 68 °C the membrane was washed with 2 x SSC, 0.1% SDS (10 x SSC is 1.5 M NaCl with 0.15 M Na-citrate) at room temperature and with 0.5 x SSC, 0.1% SDS for 15 min at 68 °C. The DIG-labeled DNA was visualized using alkaline phosphatase and a chemiluminescence substrate,

CPSD ($C_{18}H_{20}ClO_7PNa_2$; Roche) following the recommendations of the manufacturer.

PCR amplification of the DIG labeled *aehA* probe

Part of the *aehA* gene was initially cloned by PCR amplification from chromosomal DNA using the LA PCR *in vitro* cloning kit (TaKaRa Biomedicals, Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) and the following degenerated primer (pNTd) based on the N-terminal sequence of purified AEH, 5'-ATGGCNCCNGCNGCN-GAYGCNGCNCARGCNCAYGA-3' (Y=T/C; R=A/G; N=any). The PCR-products were isolated from gel (Qiaquick kit from Qiagen, GmbH, Hilden, Germany), cloned and sequenced. A gene probe for the AEH gene was made using primers based on the DNA sequence of the PCR fragment that encoded the N-terminus of AEH. The matching forward primer was 5'-CCGCTAAGCGTGCAGACCGGCAGC-3' (up-stream of pNTd) and the reverse primer was 5'-CATGCATACCGTGCCAGAACG-3'. These primers were used to amplify a 696 bp fragment (NTaehA) with Taq polymerase using the PCR DIG probe synthesis mix from Roche.

Cloning of *aehA* into an expression host

For expression of the *aehA* gene in *E. coli* the vector pETAT (*aehA* cloned in pET9) was constructed. The *aehA* gene was cloned in the *Nde*I- and *Bam*HI site of pET9 using a forward primer based on the N-terminal sequence including the leader sequence in which an *Asn*I site is incorporated, 5' CCGCCGCCG-ATTA**ATG**GTGGGACAGATTACCCTTT-3' (*Asn*I site underlined, start codon in bold) and a reverse primer in which a *Bam*HI site was incorporated (underlined), 5'-ACCCATAC-TGGATCCTTACTGTTTCACAACCGGGAG-3'. The gene was also cloned without the N-terminal leader sequence, where the leader sequence was replaced by a methionine, using 5'-GGTCGCGCATTA**ATG**GCTCCGGCAGCGGA TGC-3' (*Asn*I site underlined, start codon in bold) as a primer. After denaturation of the DNA (pLAFR3 (*aehA*)) the amplifications were established in 30 cycles of 30 s at 94 °C, 1 min at 58 °C and 1.5 min at 72 °C. Products were digested with *Asn*I and *Bam*HI and ligated into pET9 cut with *Nde*I and *Bam*HI. The ligation mixture was used to transform $CaCl_2$-competent *E. coli* BL21(DE3)pLysS. The constructs were confirmed by sequence analysis. For cloning in the *Nde*I/*Hind*III site of pEC the gene was amplified with the forward primers as described above and the following reverse primer; 5'-CATACTGGCAAGCTT**TTA**CTGTTTCACAAC CGGGAGCAG-3' (*Hind*III site underlined, stop codon in bold).

N-terminal sequence determination and protein analysis

For N-terminal sequence analysis, approximately 15 µg of protein was sliced from an SDS-PAGE gel. Eurosequence BV carried out further preparation of the sample and performed automated Edman degradation (Model 477A, Applied Biosystems).

Subunit composition determination

The subunit composition was determined via dynamic light scattering (DLS) using a DynaPro MS 80 Tc (Protein Solutions, Charlottesville, VA, USA) in combination with the Dynamics V4.0 software from Protein Solutions. A pure protein solution of 1.2 mg/ml in 50 mM Na-phosphate buffer, pH 6.2, was placed

in the laser bundle at 20 °C and data were collected for 3 min.

Inactivation

Stock solutions of the inhibitors phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl) benzenesulfonyl fluoride (Pefabloc SC) or *p*-nitrophenyl *p'*-guanidinobenzoate.HCl (*p*-NPGB) of 100 mM were made in acetonitrile, 50 mM Na-phosphate buffer (pH 6.2) and dimethylformamide, respectively. The enzyme, final concentration 2.5 µM (M_w 140 kDa), was incubated with the inhibitor for 15 min at 30 °C. Concentration of inhibitors were: PMSF, 2 mM; Pefabloc SC, 5 mM; and NPGB, 1 mM. The degree of inactivation was determined by measuring the remaining initial hydrolysis activity on NIPGB.

Enzyme assays and determination of kinetic constants

Activity of AEH was routinely assayed at 30 °C by following the hydrolysis of 15 mM NIPGB in a spectrophotometer at 405 nm in 50 mM phosphate buffer, pH 6.2 (Alkema *et al*., 2000).

The synthesis of cephalexin was followed by HPLC (Alkema *et al*., 2000). Incubations were done at 30 °C and contained 30 mM 7-ADCA and 15 mM D-PGM at pH 6.2 (50 mM Na-phosphate buffer). One cexU is defined as the amount of enzyme needed to produce one µmol of cephalexin per min. Before analysis the samples were quenched and diluted 50-fold by the addition of HPLC eluent (20 mM phosphate buffer (pH 3), 30% acetonitrile).

The initial rates (< 10% conversion) of the hydrolysis of all the substrates were determined by measuring product formation by HPLC (Alkema *et al*., 2000) except for NIPGB, which was followed as described above. The enzyme was incubated with varying concentrations in the range of 0-25 mM for cephalexin, ampicillin, HPGM and cefadroxil, or 0-50 mM for D-PGM and NIPGB, or 0-10 mM for amoxicillin. Reactions were done at 30 °C in 50 mM phosphate buffer, pH 6.2. The calculations involved non-linear regression fitting (Scientist, Micromath) using Michaelis Menten and substrate inhibition kinetics and the calculated kinetic parameters are given with their standard deviation. The hydrolysis of PGA was measured at 5 and 50 mM and the k_{cat}/K_m was calculated from the initial linear slope of the Michaelis Menten curve. Hydrolysis of glutaryl 7-ACA and adipoyl 7-ADCA was measured at 5 and 25 mM.

Nucleotide sequence accession number

The nucleotide sequence from the α-amino acid ester hydrolase has been submitted to GenBank and assigned accession no. AF439262.

RESULTS

Cloning of the gene (*aehA*) encoding the α-amino acid ester hydrolase of *A. turbidans*

To obtain an N-terminal amino acid sequence, the α-amino acid ester hydrolase (AEH) from *A. turbidans* was purified by ion- exchange, hydrophobic interaction and gelfiltration chromatography (Table 1). The native enzyme was found to be a multimer, as determined by gelfiltration, varying from a dimer to a multiple of dimers, which is in agreement with earlier observations (Ryu and Ryu, 1987). Although the yield was rather low, a small amount of pure protein of 70 kDa, in agreement with the activity peak, was obtained which was sufficient for SDS-

Table 1. Purification of α-amino acid ester hydrolase from *A. turbidans* ATCC 9325.

Purification step	Total volume (ml)	Total protein (mg)	Total activity[a] (cexU)	Specific activity[a] (cexU/mg)	Purification (fold)	Recovery (%)
Cell free extract	165	461	599	1.3	1	100
CM-sepharose	32	31	477	15	12	80
Hydrophobic interaction	5.8	1.1	68	62	48	11
Gelfiltration	2.0	0.033	22	667	513	3.7

[a] Cephalexin synthesis activity.

PAGE and amino acid sequencing (Fig 2, lane 1).

The N-terminal sequence of the 70 kDa subunit was determined as: Ala-Pro-Ala-Ala-Asp-Ala-Ala-Gln-Ala-His-Asp-Pro-Leu-Ser-Val-Gln-Thr-Gly-Ser-Asp-Ile-Pro. Based on the first 12 amino acids, and adding a starting methionine, a degenerated oligonucleotide primer (pNTd) was designed. From total DNA of *A. turbidans* a PCR product of 2.6 kb was obtained using the LA PCR *in vitro* cloning kit and pNTd. Sequence analysis of the 2.6 kb fragment indicated that the fragment contained the correct gene since downstream of the primer sequence, the DNA sequence encoded the remaining 10 amino acids of the determined N-terminus of the protein.

Sequence analysis of the *aehA* gene and its region

To ensure the completeness of the gene and to be able to study the surroundings of the *aehA* gene, a cosmid library was constructed and transduced to *E. coli*. A bank of 5670 clones with 99.9% completeness was obtained and screened with a 696 bp DIG labeled probe (NTaehA) based on part of the gene found in the 2.6 kb PCR product mentioned above. Out of the 1248 colonies screened, two hybridized with the probe. From one of these clones the cosmid was isolated and 6 kb of its insert was sequenced (Fig. 3). Four open reading frames were identified of which one harbored the determined N-terminal amino acid sequence and the sequence downstream of it found on the 2.6 kb PCR fragment. This gene (*aehA*) encoded a polypeptide with a molecular weight of 74,060, corresponding to a polypeptide of 667 amino acids. The determined N-terminal amino

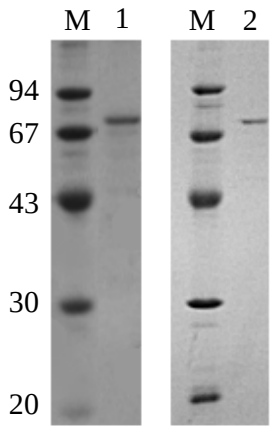
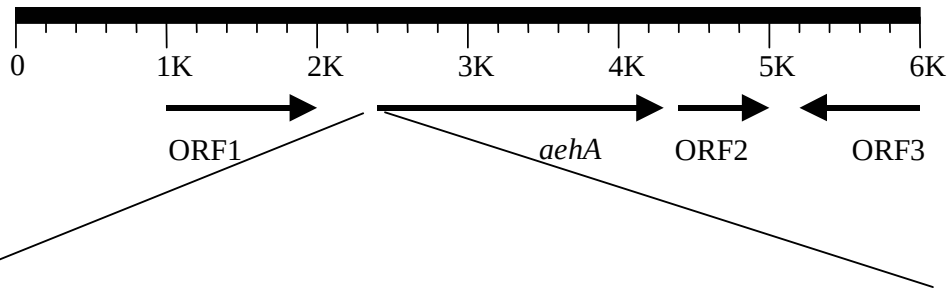


Figure 2. SDS-PAGE of α-amino acid ester hydrolase. The enzyme was purified from *A. turbidans* and *E. coli* as described in Materials and Methods. The pooled Superdex 200 fractions were analyzed by SDS-PAGE (12.5% separating and 2.5% stacking gel) and stained with Coomassie Brilliant Blue. The purified AEHs from *A. turbidans* (lane 1) and *E. coli* BL21(DE3)pLysS(pETAT) (lane 2) are shown. The band corresponding to AEH is indicated with an arrow. Molecular mass markers were loaded in lanes labeled "M", and their masses (in kilo Daltons) are shown at the left of the gels.

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   GCCGCCCGGACTGTATCGCCTGAGAATCGTTTTCTACGAAAAACAGGGTGGACGAACCGC 2220
   CTGATCCAACGCATCGTCTGAGCACCAGCTTAAAAAGCAAAAACCAGACAGAAGTGCAGG 2280
   ATCATGGTGGGACAGATTACCCTTTCAAAACAGAAATCCGTTTTGCAAAAAAAGAGCCTG 2340
1      M  V  G  Q  I  T  L  S  K  Q  K  S  V  L  Q  K  K  S  L
   TGGGCGAGCGTTGCTCTGTCCGGGGTGTTGCTGGCGGCAACTCTTCCGGTCGCGCAGGCC 2400
20  W  A  S  V  A  L  S  G  V  L  L  A  A  T  L  P  V  A  Q  A
   GCAGCTCCGGCAGCGGATGCTGCTCAGGCGCATGATCCGCTAAGCGTGCAGACCGGCAGC 2460
40  A  A  P  A  A  D  A  A  Q  A  H  D  P  L  S  V  Q  T  G  S
   GATATTCCGGCCAGTGTGCATATGCCAACAGACCAGCAGCGTGATTACATCAAGCGCGAG 2520
60  D  I  P  A  S  V  H  M  P  T  D  Q  Q  R  D  Y  I  K  R  E
   GTTATGGTCCCCATGCGGGACGGCGTGAAGCTGTATACGGTTATTGTTATTCCGAAAAAC 2580
80  V  M  V  P  M  R  D  G  V  K  L  Y  T  V  I  V  I  P  K  N
   etc.
```

Figure 3. Genetic organization around *aehA* and the N-terminal part of the protein and its nucleotide sequence. The deduced amino acid sequence is shown below the nucleotide sequence. The N-terminal sequence found by amino acid sequencing of the mature wild-type protein is underlined, the putative ribosome binding site is shown in bold. The open reading frames are described in the text.

acid sequence was found at position 41-62 (Fig. 3), indicating that the first 40 amino acids of the protein were cleaved off during maturation in *A. turbidans.*

The genetic organization of the DNA region in which the *aehA* gene is situated could give indications about the biological function of the AEH. Identification of open reading frames surrounding the ORF of the *aehA* gene was possible (Fig. 3) after a search for sequence similarity in the non-redundant database at NCBI using Blastx (Altschul *et al*., 1990). Upstream of the *aehA* gene ORF1 was detected which has 56% identity to a phosphoserine aminotransferase from *Methanosarcina barkeri* (Metcalf *et al*., 1996). Downstream of the *aehA* gene a putative protein of 30 kDa (ORF2) was found which shows 22% identity with part of the creatinine amido hydrolase of *Bacillus halodurans* (protein ID no. BAB03945) (Takami *et al*., 2000). Further downstream, in the opposite direction, the C-terminal part of a protein (ORF3) was found which has significant identity (50%) to a succinicsemialdehyde dehydrogenase from *Deinococcus radiodurans* (protein ID no. BAA21377).

Database searches using Blast (Altschul *et al*., 1990) indicated that the deduced amino acid sequence of *aehA* showed homology with several (putative) proteins, most of which originated from genome sequencing projects and have an unknown function (Table 2, Fig. 4). The most closely

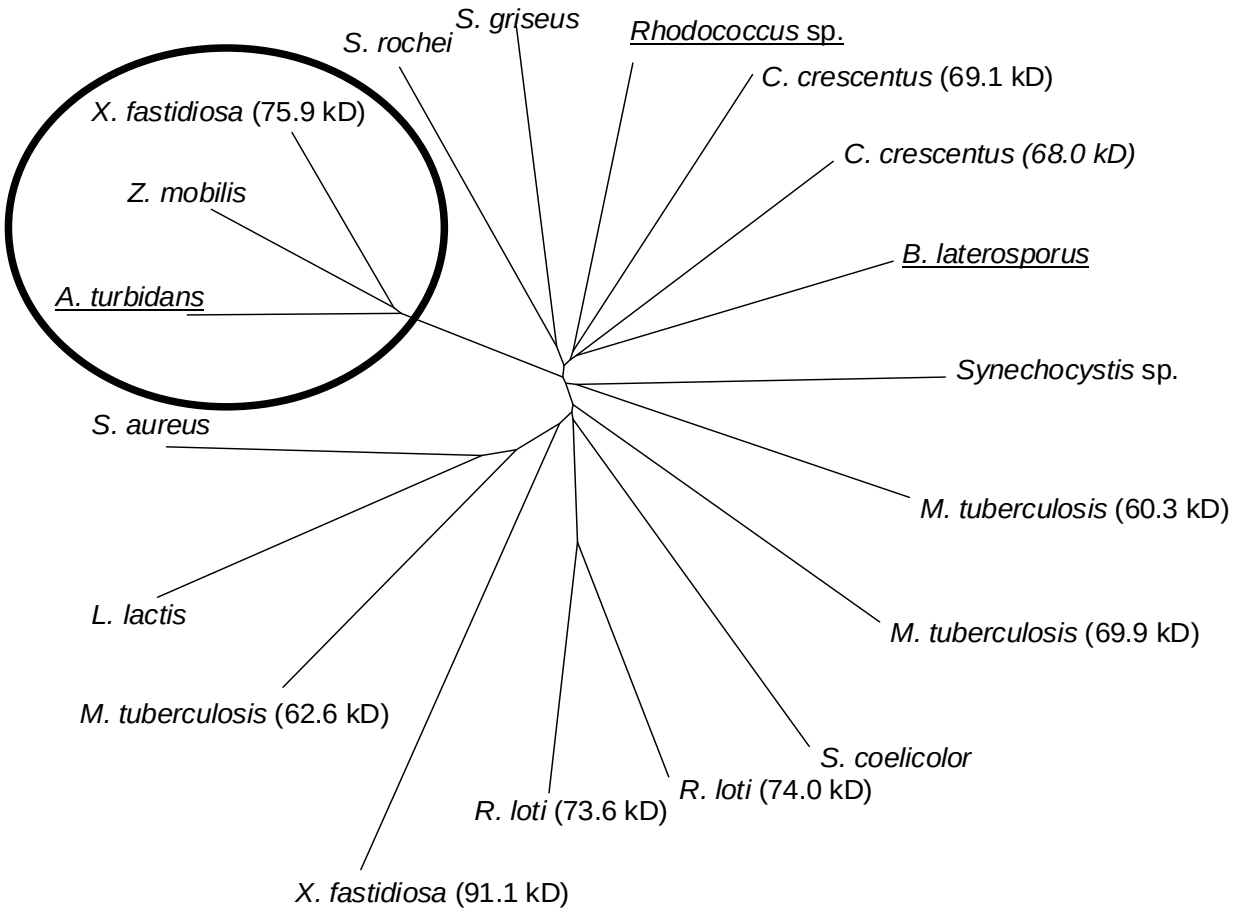

Figure 4. A dendrogram of proteins that are homologous to the α-amino acid ester hydrolase of *A. turbidans* as found by BLAST search. The distance can be read as number of nucleotide substitutions per site. The proteins that share more than 60% identity with AEH are encircled. The proteins with described activity are underlined. The tree was constructed using Clustal W and TreeView.

related protein, for which the activity is described, is the intracellular cocaine esterase from the gram-positive strain *Rhodococcus* sp. strain MB1 (Bresler *et al.*, 2000). This enzyme hydrolyzes the ester bond in cocaine resulting in benzoate and ecgonine methyl ester. The next most related studied enzyme is the glutaryl-7-ACA acid acylase of *Bacillus laterosporus* that hydrolyzes glutaryl-7-ACA acid to 7-aminocephalosporanic acid. Activity of these enzymes for α-amino esters or β-lactam antibiotics carrying an α-amino acid acyl side chain has not been reported. The glutaryl-7-ACA acid acylase is composed of a polypeptide with molecular size of 70 kDa, which corresponds to the size of the subunits found for AEH from *A. turbidans* (this study).

An extended search for homologous proteins using the position-specific iterated Blast program PSI-Blast (Altschul *et al.*, 1997) indicated low identity (average 14%) to X-prolyl dipeptidyl aminopeptidases from *Lactococcus* and *Lactobacillus* strains. The X-prolyl dipeptidyl aminopeptidases belong to the peptidase_S15 family as defined by the Pfam database (Bateman *et al.*, 2000). These enzymes are serine proteases with the active-site serine located in the consensus sequence GxSYxG, where x is a non-conserved amino acid (Chich *et al.*, 1992). The AEH shows

conservation of this motif and its direct surroundings (Fig. 5). However, there is no significant overall similarity, which makes it impossible to judge if the proteins are structurally related.

Expression in *E. coli* and properties of the recombinant protein

The cosmid clones that harbor the *aehA* gene and about 20 kb surrounding DNA did not show any AEH activity, indicating that the enzyme was not expressed properly. The complete *aehA* gene of 2004 bp was subcloned in the expression vectors pEC and pET9 which resulted in active constructs that were designated pAT and pETAT, respectively. This confirms that AEH is encoded by *aehA* and indicates that probably the bad positioning of the ribosome binding site (Fig. 3) or an unrecognized promoter caused the lack of expression from the cosmids in *E. coli*. Although overexpression was limited, due to the increased culture densities and the improved purification method, 11 times higher quantities of pure protein, (Fig. 2, lane 2) per liter of culture were obtained from *E. coli* BL21(DE3)pLysS(pETAT) (Table 3) compared to the natural host.

As described above, the *aehA* gene codes for a precursor protein with an N-terminal leader sequence of 40 amino acids and the 70 kDa subunit of AEH. The sequence of the first 40 amino acids has features typical for signal peptides, like positively charged residues at the N-terminus followed by a stretch of hydrophobic residues (von Heijne, 1985). In the carboxy-terminal segment of the leader sequence, a consensus pattern specific for the cleavage by signal peptidase I (AXA) is present (Nakai, 2000; Nakai and Horton, 1999; Nielsen *et al*., 1997). This, however, predicts the cleavage site at position 39-40 (AQA-AAP) which is one position earlier than what was found by N-terminal amino acid sequencing of the enzyme (40-41, AQAA-AP). To obtain insight in the function of the leader sequence and localization of the enzyme, the osmotic shock procedure was performed on the wild-type organism and the clones. Moreover, constructs without the leader sequence were made. The osmotic shock procedure developed for *E. coli* (Alkema *et al*., 1999) was used with *A. turbidans*, but no released proteins were detected. In contrast, the same procedure released DNA from *E. coli* BL21(DE3)pLysS(pETAT),

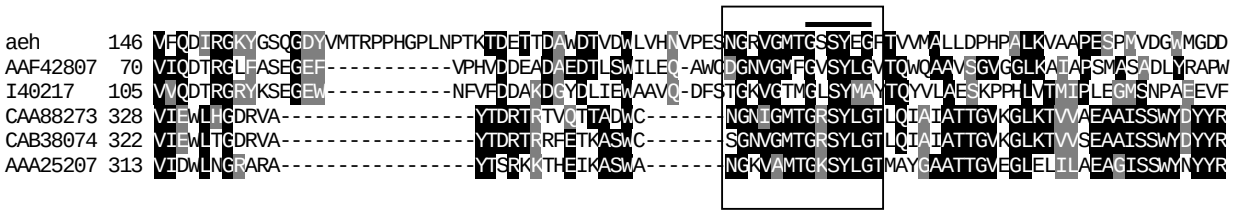

Figure 5. Partial alignment of α-amino acid ester hydrolase (AEH) from *A. turbidans* with other (putative) serine hydrolases. Sequences: glutaryl-7-ACA-acylase precursor from *B. laterosporus* (protein ID no. I40217), cocaine esterase from *Rhodococcus* sp. (protein ID no. AAF42807), X-prolyl dipeptidyl aminopeptidases from *Lactococcus lactis* (protein ID no. AAA25207), *Lactobacillus delbrueckii* (protein ID no. CAB38074) and *Lactobacillus helveticus* (protein ID no. CAA88273). The alignment was performed using the pattern induced (local) multiple alignment (PIMA 1.4) facilitated by the BCM search launcher on the World Wide Web. The region of relatively high identity among all 7 proteins is boxed. The bold line indicates the GxSYxG motif.

Table 2. Amino acid sequence similarities of the α-amino acid ester hydrolase of *A. turbidans* ATCC 9325.

Protein ID[a]	Organism	Function	Identity[b] (%)	Mw (kDa)	Motif
AF439262	*Acetobacter turbidans*	AEH precursor (this study)	100	74.0	**G**S**SY**E**G**
AAD29644	*Zymomonas mobilis*	n.d.[d]	60	73.4	**G**S**SY**E**G**
AAF83839	*Xylella fastidiosa*	n.d.	61	75.9	**G**S**SY**E**G**
AAF42807	*Rhodococcus* sp.	cocaine esterase [*]	29	62.1	**G**V**SY**L**G**
AAK23601	*Caulobacter crescentus*	n.d.	27	68.0	**G**L**SY**GA
AAK22924	*Caulobacter crescentus*	n.d.	28	69.1	**G**C**S**SSA
BAA10148	*Bacillus laterosporus*	glutaryl 7-ACA acylase precursor [#]	26	72.2	**G**L**SY**MA
BAA17073	*Synechocystis* sp.	n.d.	25	60.0	**G**F**SY**Q**G**
CAB67720	*Streptomyces rochei*	n.d.	24	58.1	**G**R**SY**EA
AAC26133	*Streptomyces griseus*	n.d.	26	61.6	**G**T**SY**QA
CAB03646	*Mycobacterium tuberculosis*	n.d.	26	60.3	**G**LP**Y**L**G**
CAB53331	*Streptomyces coelicolor*	n.d.	25	69.9	**G**K**SY**DA
CAB07817	*Mycobacterium tuberculosis*	n.d.	28	62.6	**G**S**SY**LA
CAB01467	*Mycobacterium tuberculosis*	n.d.	23	69.9	**G**N**SY**D**G**
BAB53185	*Rhizobium loti*	n.d.	22	74.0	**G**I**S**WG**G**
BAB51629	*Rhizobium loti*	n.d.	22	73.6	**G**I**S**WG**G**
AAK05333	*Lactococcus lactis*	n.d.	27	64.9	**G**T**SY**LA
BAB43685	*Staphylococcus areus*	n.d.	26	64.4	**G**V**SY**LA
AAF82828	*Xylella fastidiosa*	n.d.	38[c]	91.1	**G**G**SY**G**G**

[a] Protein ID gives the accession number of the protein database of NCBI.
[b] The percentage of identity is determined using the pairwise alignment option of Blast with default settings of the parameters.
[c] This identity was found in a stretch of 100 residues.
[d] n.d., not described
[*] (Bresler *et al*., 2000)
[#] (Aramori *et al*., 1991b)

indicating that the cell wall of this strain is not rigid enough, which is ascribed to the presence of lysozyme, coded by pLysS. A periplasmic extract from *E. coli* HB101(pAT) was made but no AEH activity was detected. A control experiment using *E. coli* HB101(pEC), expressing the periplasmic penicillin acylases from *E. coli* ATCC 11105, showed that the procedure worked. Cloning of the *aehA* gene in *E. coli* without the leader sequence,

Table 3. Purification of α-amino acid ester hydrolase from *E. coli* BL21(DE3)pLysS(pETAT).

Purification step	Total volume (ml)	Total protein (mg)	Total activity (cexU)	Specific activity[a] (cexU/mg)	Purification (fold)	Recovery (%)
Cell free extract	184	1174	2348	2	1	100
DEAE	195	60	2050	34	17	87
CM-Hap	80	5.8	1109	191	96	47
Hydrophobic interaction	32	1.0	633	605	303	27
Gelfiltration	6.6	0.18	153	859	430	7

[a] Cephalexin synthesis activity.

starting with M-41-APAAD, resulted in inactive clones, using two different expression systems (pET9, pEC). To determine the way of processing in *E. coli* BL21(DE3)pLysS(pETAT), the first 5 N-terminal amino acid residues of the recombinant enzyme were determined. Multiple signals were present but the major sequence found was 40-AAPXAD, which is in agreement with the predicted cleavage site between residues 39 and 40. This indicates that the leader sequence is processed in a similar way in *E. coli* as it is in *A. turbidans.* The other signals present indicate some N-terminal heterogeneity, which might be caused by cleavage at the other potential peptidase I cleavage sites (position 41-49, Fig. 3) or by non-specific protease activity.

Characterization and kinetic properties of recombinant AEH

The subunit composition of the recombinant purified enzyme was determined via dynamic light scattering. The average of two measurements resulted in a molecular weight of 150 kDa, which is in good agreement with a dimeric form of the enzyme (140 kDa).

To study the substrate specificity of the recombinant AEH, steady state kinetic parameters were determined for a range of substrates (Table 4). For most compounds Michaelis-Menten kinetics was observed. Substrate inhibition was confirmed in the hydrolysis of ampicillin (Takahashi *et al.*, 1974) and also observed for the chromogenic substrate NIPGB. The V versus [S] curves could be fitted according to the common equations given for substrate inhibition. The K_m values for cephalexin and D-PGM obtained with the recombinant AEH were in reasonable agreement with values given in the literature for the enzyme from *A. turbidans*, respectively 1.5 mM and 4.9 mM (Ryu and Ryu, 1988; Takahashi *et al.*, 1974) (Table 4). The AEH did not display detectable activity towards glutaryl 7-ACA, which is a substrate for the related glutaryl 7-ACA acylase from *B. laterosporus*. Adipoyl 7-ADCA was not hydrolyzed by AEH either. In general we see for esters a high k_{cat} in combination with a high K_m. The addition of an OH group on the aromatic moiety of the acyl group reduces the activity. This shows that the nature of the acyl group has a large influence on the activity.

Table 4. Kinetic parameters of α-amino acid ester hydrolase.

Substrate[a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.mM^{-1}$)
NIPGB[b]	1.1 ± 0.5	0.4 ± 0.07	0.4 ± 0.2
Cephalexin	0.34 ± 0.03	347 ± 10	1021 ± 95
Ampicillin[b]	1.0 ± 0.6	162 ± 61	162 ± 115
D-PGM	7 ± 2	1035 ± 123	148 ± 46
D-PGA[c]	>13	>43	3.3
Cefadroxil[d]	1.7 ± 0.3	9.6 ± 0.4	6 ± 1
Amoxicillin[d]	2.6 ± 0.2	10 ± 0.3	3.9 ± 0.3
HPGM	11 ± 3	263 ± 30	24 ± 7

[a] For glutaryl 7-ACA and adipoyl 7-ADCA the hydrolysis was less than 300 $nmol.s^{-1}.mg^{-1}$ of AEH at 25 mM substrate.
[b] Substrate inhibition was observed with K_i of 200 mM (NIPGB) and K_i of 3 mM (Ampicillin).
[c] k_{cat}/K_m was calculated from the initial slope of the Michaelis Menten curve, the error is 30%.
[d] Data were measured using His-tagged protein with the same catalytic properties as wild type (to be published).

Inhibition

The conservation of the GxSYxG motif suggests that AEH is a serine hydrolase. To explore this possibility some known serine protease/hydrolase inhibitors, Pefabloc SC, PMSF and *p*-NPGB, were tested. Incubation with Pefabloc SC resulted in a 10% loss in activity and PMSF did not give inactivation at all. Significant reduction (75%) of the initial enzyme activity was observed using *p*-NPGB. This suggests that a serine is involved in the catalytic mechanism.

DISCUSSION

Almost 30 years ago the α-amino acid ester hydrolases were first described and they have since been repeatedly explored for use in biocatalysis, but no amino acid sequence information has been reported so far. We cloned the gene encoding the α-amino acid ester hydrolase (AEH) from *A. turbidans* out of a cosmid library via Southern blotting. From the literature it was expected that the gene for AEH would code for two different subunits, one of 70 and one of 72 kDa (Ryu and Ryu, 1987; Takahashi *et al.*, 1974). However, only one gene (*aehA*) coding for a protein of 74 kDa was found. Since the determined N-terminal sequence was found at position 41 it was concluded that *aehA* encodes a precursor of AEH that undergoes processing to yield an active enzyme of 70 kDa. No evidence for a second subunit of 72 kDa was obtained, neither by purification (Fig. 2) nor by sequence analysis (Fig. 3). However, incomplete processing of the leader sequence might have caused the presence of two apparently different subunits of 70 and 74 kDa, which could easily have been interpreted as subunits of 70 and 72 kDa.

Expression of the single *aeh*-gene in *E. coli*, including the identified leader sequence, produces active dimeric AEH. Processing of the

leader sequence in *E. coli* implies a periplasmic localisation, since signal peptidase I is active on the periplasmic face of the cytoplasmic membrane (Arkowitz and Bassilana, 1994). However the enzyme was not released from the *E. coli* HB101(pAT) cells by a standard osmotic shock procedure. It is therefore assumed that the protein sticks to cell envelope. Since no activity was detected when the enzyme was expressed without the leader sequence, it is proposed that the 40 amino acid N-terminal part facilitates proper folding of the enzyme. Overall, it can be concluded that the N-terminal sequence is needed for production of active enzyme and probably serves for transport to the periplasm.

The deduced amino acid composition of *aehA* from *A. turbidans* is similar to the experimental data published in the literature, except for the number of methionines and cysteines, for which we find significantly less (Ryu and Ryu, 1987). Nevertheless, the deduced values for these amino acids are in reasonable agreement with the experimental data found for both *X. citri* and *P. melanogenum* (Kato *et al*., 1980a; Kim and Byun, 1990). This suggests a high similarity between the different AEHs, as expected from their comparable catalytic and structural properties.

Database searches with the *aehA* encoded protein revealed no homology with any other known penicillin or cephalosporin acylase, apart from the glutaryl 7-ACA acylase of *B. laterosporus*. However, glutaryl 7-ACA was not hydrolyzed by the AEH of *A. turbidans*, which is probably due to the absence of an amino group on the Cα position (Ryu and Ryu, 1988; Takahashi *et al*., 1974). The conservation of the GxSYxG motif in AEH, cocaine esterase (Bresler *et al*., 2000) and other putative acylases (Table 2) suggests that AEH, is a serine hydrolase, which was further indicated by the inactivation by *p*-NPGB. In glutaryl 7-ACA acylase the GxSYxG motif is not fully conserved, the last glycine is replaced by an alanine (GLSYMA, Table 2). This might indicate that the second glycine influences the substrate range.

The physiological role of the β-lactam antibiotic acylases has not been elucidated yet. It has been suggested that penicillin G acylase from *E. coli* is involved in the degradation of phenylacetylated compounds for the generation of phenylacetic acid as a carbon source (Valle *et al*., 1991). Although the *aehA* gene appears to be located in an area where genes involved in the metabolism of amino compounds are situated, the real function of the AEH remains unclear. Further investigation of the substrate range of the AEH might reveal a relation to the surrounding enzymes.

Comparison of some kinetic values of the cloned AEH to literature data showed that the recombinant AEH has similar kinetic properties as the wild-type enzyme (Ryu and Ryu, 1988; Takahashi *et al*., 1974). Remarkable features are the better esterase than amidase activity with related substrates (D-PGM compared to D-PGA) and the need for an α-amino group. The higher specificity for the acyl donor compared to the corresponding antibiotic in the case of cefadroxil and amoxicillin are favorable for high product accumulation in a synthesis reaction. Even though esters are generally preferred, the specificity constant for cephalexin is higher than for D-PGM. This is unexpected from the classification of AEH as an esterase. Therefore, a broader exploration of the substrate range is needed.

The AEH of *A. turbidans* was classified, based on the preferred antibiotic substrate, as an

ampicillin acylase (Savidge, 1984; Vandamme and Voets, 1974). However, cephalexin is the preferred β-lactam antibiotic for AEH, as described for other AEHs as well (Blinkovsky and Markaryan, 1993; Kato *et al.*, 1980b; Kim and Byun, 1990; Ryu and Ryu, 1988), and the enzyme should therefore be designated as a cephalexin acylase.

X-ray analysis and mutational studies have shown that β-lactam antibiotic acylases from different substrate specificity classes all belong to the Ntn-hydrolase superfamily (Brannigan *et al.*, 1995; Kim *et al.*, 2000; Lee *et al.*, 2000; Suresh *et al.*, 1999). Since both AEH and the glutaryl 7-ACA acylase from *B. laterosporus* (Aramori *et al.*, 1991b) do (i) not have an N-terminal serine, threonine or cysteine, (ii) contain the serine protease motif and, (iii) show no homology with any other known penicillin acylases (Fig. 4), it is very unlikely that these enzymes belong to the superfamily of Ntn-hydrolases. We could not identify homologous proteins with a known X-ray structure that might reveal a structural relation of AEH to other protein families. Therefore we conclude that AEH, together with the glutaryl 7-ACA acylase from *B. laterosporus*, represents a new class of β-lactam antibiotic acylases, each representing a different subclass.

The expression of the AEH from *A. turbidans* ATCC 9325 in *E. coli* makes it possible to study the enzyme in more detail. The catalytic mechanism and the structural features that both determine the biocatalytic performance will be investigated further in order to gain more insight in the structure-function relationship of this new class of acylases.

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4

Identification of the catalytic residues of α-amino acid ester hydrolase from *Acetobacter turbidans* by labeling and site-directed mutagenesis

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ABSTRACT

The α-amino acid ester hydrolase (AEH) from *Acetobacter turbidans* ATCC 9325 is capable of hydrolyzing and synthesizing the side chain peptide bond in β-lactam antibiotics. Database searches revealed the presence of an active site serine consensus sequence Gly-x-Ser-Tyr-x-Gly that is also found in X-prolyl dipeptidyl aminopeptidase. The serine hydrolase inhibitor *p*-nitrophenyl-*p*'-guanidino-benzoate appeared to be an active site titrant and was used to label the α-amino acid ester hydrolase. Electrospray MS and ES/MS/MS analysis of peptides from a CNBr digest of the labeled protein showed that Ser205, situated in the consensus sequence, becomes covalently modified by reaction with the inhibitor. Extended similarity searches showed alignment of this Ser205 with the catalytic nucleophile of some α/β-hydrolase fold enzymes, which posses a catalytic triad composed of a nucleophile, acid and base. Based on the alignments, 10 amino acids were selected for site-directed mutagenesis (Arg85, Asp86, Tyr143, Ser156, Ser205, Tyr206, Asp338, His370, Asp509 and His610). Mutation of Ser205, Asp338 or His370 to an alanine almost fully inactivated the enzyme, whereas mutation of the other residues did not seriously affect the enzyme activity. Circular dichroism measurements showed that the inactivation was not caused by drastic changes in the tertiary structure. Therefore, we conclude that the catalytic domain of AEH has an α/β-hydrolase fold structure with a catalytic triad of Ser205, Asp338 and His370. This distinguishes AEH from other known β-lactam antibiotic acylases.

INTRODUCTION

The α-amino acid ester hydrolase have been known for their applicability in the biocatalytic synthesis of semi-synthetic β-lactam antibiotics since 1972 (Takahashi *et al.*, 1972). These enzymes can couple activated side chains to β-lactam nuclei. Interesting features of these enzymes are the ability to accept charged substrates, the preference for esters over amides and the low pH-optimum (pH 6.2) (Blinkovsky and Markaryan, 1993; Takahashi *et al.*, 1974). Despite these attractive properties, the gene encoding the α-amino acid ester hydrolase (AEH) of *A. turbidans* was only recently cloned and characterized (Polderman-Tijmes *et al.*, 2002a). Thus far, all the known β-lactam antibiotic acylases, such as penicillin G acylase (Duggleby *et al.*, 1995), penicillin V acylase (Suresh *et al.*, 1999) and cephalosporin acylase (Kim *et al.*, 2000), belong to the Ntn-hydrolase family. However, protein database searches showed no homology of AEH with known β-lactam antibiotic acylases. The N-terminal amino acid sequence of AEH was determined which revealed a signal sequence but no N-terminally located Thr, Ser or Cys, characteristic for members of the Ntn-hydrolase superfamily. It was therefore postulated that the AEHs belongs to a new class of β-lactam antibiotic acylases (Polderman-Tijmes *et al.*, 2001).

An alignment of the AEH sequence with that of homologous proteins showed the presence of the active site serine consensus motif GxSYxG (Polderman-Tijmes *et al.*, 2002a), which is described for the X-prolyl dipeptidyl aminopeptidases (Chich *et al.*, 1992). At present, no X-ray structure of the aminopeptidases is

available, but they are members of a group of proteins that belongs to the prolyl oligopeptidase family. Of this family two structures have been solved which both contain an α/β-hydrolase fold (Fülöp *et al*., 1998; Medrano *et al*., 1998) and have a catalytic triad of Ser, Asp and His. Therefore, it is possible that the X-prolyl dipeptidyl aminopeptidases and hence AEH also have a catalytic triad. This assumption is further supported by the identification of a catalytic triad in the recently solved crystal structure of a cocaine esterase (Larsen *et al*., 2001) that is also related to AEH. Earlier experiments with inhibitors already suggested the importance of a histidine for the catalytic activity of AEH (Ryu and Ryu, 1988). However, common serine hydrolase inhibitors such as phenylmethyl-sulfonyl fluoride, di-isopropylfluorophosphate or Pefabloc SC showed no inhibition of AEH activity (Polderman-Tijmes *et al*., 2002a; Ryu and Ryu, 1988). On the other hand, inhibition was observed with the serine hydrolase inhibitor *p*-nitrophenyl-*p*'-guanidino-benzoate (*p*-NPGB) (Polderman-Tijmes *et al*., 2002a). However, the inhibition was incomplete which left uncertainty about the catalytic role of a serine in AEH. In this study we used active-site labeling, site-directed mutagenesis, and sequence analysis to demonstrate that AEH is a member of a class of β-lactam antibiotic acylases that belongs to the α/β-hydrolase fold family and possesses a classical catalytic triad of Ser, Asp and His.

MATERIAL AND METHODS

Materials

The chromogenic substrate D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) was obtained from Syncom (Groningen, The Netherlands). Phenylglycine methyl ester (PGM), 7-aminodesacetoxycephalosporanic acid (7-ADCA), and cephalexin were provided by DSM Anti-infectives (Delft, the Netherlands). All chemicals used in DNA manipulation procedures were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and used as recommended by the manufacturer. The oligonucleotides for the cloning of the *aehA* gene and introduction of point mutations were synthesized by Eurosequence B.V. (Groningen, the Netherlands).

Bacterial strains, plasmids and growth conditions

E. coli TOP10 (Invitrogen, Breda, The Netherlands) was used for cloning derivatives of pBAD/*Myc*-HisA (Invitrogen) and pTrcHisB (Invitrogen). *E. coli* strain BL21(DE3)pLysS (Promega corporation, Madison, USA) was used for cloning derivatives of pET28 (Promega). The *E. coli* strains were grown at 30 °C for plasmid isolation. For expression, strains with pTrcHisB and pET28 derivatives were grown on LB medium at 30 °C and directly induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.4 mM). The antibiotics ampicillin and kanamycin were added to the media at 100 µg/ml and 50 µg/ml, respectively.

Molecular cloning

To clone *aehA* in the *Nco*I and *Hind*III site of pBAD/*Myc*-HisA, resulting in pBADAT, the *Nco*I restriction site was first removed from the gene cloned in pAT (Polderman-Tijmes *et al*., 2002a. This was accomplished by PCR using the sense primer 5'-GAACTGCCTGTGTCT-ATGGATATTTTCCGGGGC-3', the compatible reverse complement primer and the QuickChange site-directed mutagenesis kit of Stratagene (La Jolla, USA) resulting in pATdelNco. From this

construct the gene encoding AEH was amplified by PCR using two mutagenic primers to allow cloning in the *Nco*I and *Hind*III site of pBAD/*Myc*-HisA. The forward primer, 5'-CGCGCCACACC**ATGG**T-GGGACAGATTA-3' (start codon in bold), was based on the N-terminal sequence including the signal sequence and an *Nco*I site (underlined) was introduced. The reverse primer, 5'-CATACTGGCAAGCTTCTGTTT-CACAACCGGGAG-3' (*Hind*III site is underlined), lacked the stop codon to allow the C-terminal attachment of the *myc*-epitope followed by a polyhistidine region of 6 histidine residues (His_6-tag) encoded on pBAD/*Myc*-HisA.

The *aehA* gene was cloned in the *Nco*I and *Hind*III site of pET28 (Promega corporation, Madison, USA) by the same procedure as described above, resulting in direct C-terminal attachment of a nucleotide sequence encoding six histidine residues.

Site-directed mutagenesis and sequencing

Site-directed mutagenesis was performed on pBADAT using the QuickChange site-directed mutagenesis kit of Stratagene (La Jolla, CA, USA) according to the procedure recommended by the manufacturer. When possible, a restriction site was introduced in the mutagenic primers (Table 1). The PCR reaction mixture was directly used to transform chemically competent *E. coli* TOP10 cells. For isolation of vector the cultures were grown overnight on LB medium at 30 °C. Mutated plasmids where checked by restriction analysis (when possible). All mutants and constructs were verified by DNA sequencing at the Department of Medical Biology of the University of Groningen (Groningen, The Netherlands).

Table 1. Synthetic oligonucleotides

Oligonucleotide sequence 5' → 3'	Restriction site	Amino acid substitution
C GAG GTT ATG GT**A** CCC ATG **GC**G GAC GGC GTG AAG CTG	*Rsa*I	Arg85Ala
GTT ATG GT**A** CCC ATG CGG G**C**C GGC GTG AAG CTG	*Rsa*I	Asp86Ala
GC GGG AAA TAT GGC **G**CT CAG GGC GAT TAT G	*Hae*II	Ser156Ala
GGT ATG ACA GG**G** TCG **G**CC TAT GAG GGC TTT ACT	*Asp*I	Ser205Ala
G GGT ATG ACA GG**G** TCG TCC **GC**T GAG GGC TTT ACT G	*Asp*I	Tyr206Ala
GAA CAG GGC TTG TGG G**C**T CAG GAA GAT ATG TG	-	Asp338Ala
G ATG GGC CC**A** TGG CGG **GC**T AGT GGG GTG AAC	*Nco*I	His370Ala
G TTT GTA GAG GGC GGC **GC**T ATC CGC GTG TTT CAG	-	Tyr143Ala
CA GAA TCC CGC CCG G**C**T GTG GTG AC**A** TAT GAA AC	*Nde*I	Asp509Ala
C CAT GTG TTT GCA AAA GGG **GC**T CGG ATT ATG GTG CAG	-	His610Ala

Oligonucleotides used in site-directed mutagenesis. Only the sense primers are shown. Introduced restriction sites are underlined, sequence differences with wild type are shown in bold.

Protein purification

Wild-type and mutated AEHs were expressed in *E. coli* TOP10 from the pBAD/*Myc*-HisA derived constructs. Expression of the mutants was tested at different arabinose concentrations (0.1, 0.01, 0.001 and 0.0001%) and at different temperatures 14 °C and 18 °C and 30 °C. The optimal conditions were used for large-scale preparations. To obtain soluble protein two 2.5 l cultures supplemented with l-arabinose (0.01% w/v) were inoculated with a 1 ml overnight culture grown at 30 °C and incubated for 64 h at 14 °C. Induced cells were harvested from the cultures by centrifugation at 5000 g and suspended in 50 mM Na-phosphate buffer pH 6.2. All further steps were carried out at 4 °C. The cytoplasmic content was released by sonification and the remaining cell debris was removed by centrifugation at 13,000 g for 40 min. The supernatant was added to 1 ml Ni-agarose (Qiagen) equilibrated with wash buffer (25 mM imidazole, 500 mM NaCl, 50 mM Na-phosphate, pH 7.4). After mixing by inversion for 90 min at 4 °C the bed was allowed to form (20 x 8 mm in a polyprep chromatography column (Bio-Rad Laboratories, Hercules, CA, USA)). The unbound protein was washed from the column with 30 column volumes of wash buffer. The bound protein eluted from the column at 75-100 mM imidazole in a stepwise gradient from 50 to 200 mM imidazole, 150 mM NaCl, 50 mM Na-phosphate, pH 7.4 in 20 column volumes. The protein was brought to 50 mM Na-phosphate buffer pH 6.2 with use of an Econo-Pac gelfiltration column (Bio-Rad). All purification steps were monitored by SDS-PAGE and the enzymatic activity was measured with NIPGB (Polderman-Tijmes *et al.*, 2002a). The protein concentrations were measured using the Bradford method with bovine serum albumin as the standard.

Analysis of conformation by CD spectroscopy

Far-UV CD spectra from 250 to 190 nm were recorded on an AVIV circular dichroism spectrometer model 62A DS (AVIV Associates, Lakewood, NJ, USA) at 25 °C using a quartz cuvette with a path length of 0.1 cm. The concentration of wild-type and mutant enzymes was 0.2 mg/ml in 50 mM Na-phosphate buffer, pH 6.2. Per sample three separate spectra were collected and averaged using a step interval of 0.5 nm/min and an averaging time of 5 s. The phosphate buffer was used as a blank and subtracted from each recording. The data was converted to mean residue ellipticity (θ_{MRE}, $deg.cm^2.dmol^{-2}$). From the CD spectra the percentage of secondary structure elements was calculated using CD spectra deconvolution (CDNN version 2.1, available on the World Wide Web). These values were standardized to 100% total structure elements.

Activity assays

The hydrolysis and synthesis of cephalexin at 30 °C was followed by high-pressure liquid chromatography (HPLC) as described before (Polderman-Tijmes *et al.*, 2002a). The hydrolysis of *p*-NPGB was measured at concentrations varying from 0.1 to 1 mM with 1.5 µM enzyme. The release of *p*-nitrophenol (*p*-NP) was measured at 405 nm and 30 °C using a spectrophotometer (Lambda Bio 10 and software package UV WinLab, Perkin Elmer, Norwalk, USA). A stock solution of *p*-NPGB (10 mM) was made in dimethylformamide (DMF) and acetonitrile (ACN) in a 1:4 volume ratio. The steady state reactions were done in 50 mM Na-phosphate

buffer, pH 7.0. The molar extinction coefficient of *p*-NP at pH 7 was determined as 9200 M^{-1} cm^{-1}.

The pre-steady state kinetics of *p*-NPGB conversion was determined using an Applied Photophysics SX17MV stopped-flow instrument. A stock solution of *p*-NPGB (100 mM) was made in DMF. The final concentration of DMF in the reaction mixture was 2% or lower. All pre-steady state reactions were performed in 50 mM 4-morpholinepropane-sulfonic acid buffer at pH 7, with 1 mM *p*-NPGB. The enzyme concentration used was 1.32 or 0.66 µM (α_2; 144 kDa). Progress curves were fit to equation 1 to obtain the amplitude and the first order rate constant for the burst phase and the velocity of the steady-state reaction, using the program Scientist.

(1) $$[P_1] = A.t + B(1-e^{-k'.t})$$

Inactivation and reactivation of AEH-His

The enzyme (2.4 µM, 144 kDa) was inactivated by incubation with *p*-NPGB (1 mM, 1% DMF) for 15 min at 30 °C. Control experiments involved incubation under the same conditions of solely enzyme and enzyme with 1% DMF. To study reactivation, the inactivated enzyme was diluted 76 fold in 15 mM NIPGB dissolved in 50 mM Na-phosphate, pH 6.2. The time course of reactivation was monitored by following the hydrolysis of NIPGB at 30 °C and 405 nm.

Labeling of the enzyme

The enzyme was incubated with *p*-NPGB in 50 mM Na-phosphate buffer, pH 6.2, with 0.5% dimethylformamide, for 15 min at 30 °C. The excess *p*-NPGB was removed by dialysis against 70% formic acid. To reduce any disulfide bonds the enzyme solution was dialyzed against 70% formic acid with β-mercaptoethanol (2 mM). After removing the β -mercaptoethanol by dialysis against 70% formic acid, the labeled protein was treated with a 100-fold molar excess of CNBr over the Met content. The reaction was allowed to proceed for 24 h at room temperature under N_2 in the dark and was stopped by addition of 10 volumes of water. The reaction mixture was freeze-dried and dissolved in HPLC eluens. The generated peptides were separated by reversed-phase HPLC using a Nucleosil-5 C18 column (4.6 by 300 mm, Alltech) at 1 ml/min in a linear gradient of 0 to 67% acetonitrile in 0.1% trifluoroacetic acid. The peptide profile was monitored at 280 nm. The control experiment involved the same conditions as described above except that no *p*-NPGB was added. The peaks that were different from the control experiment were collected and rechromatographed on the same column in a linear gradient from 0-67% acetonitrile in 0.1% ammonium acetate, pH 5.0. The individual peaks were collected, concentrated and injected directly into the mass spectrometer.

Mass spectrometry

Electrospray mass spectrometry (ES/MS) was performed on an API3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada), a triple quadrupole mass spectrometer supplied with an atmospheric pressure ionization source, and an ionspray interface (Bruins, 1991). The spectra were scanned in the range between *m/z* 400 and 1600. MS/MS product ion spectra were recorded on the same instrument by selectively introducing the *m/z* 1229.5 (singly charged unlabeled peptide) and *m/z* 695.9 (doubly charged labeled peptide) precursor ions from the first quadrupole into the collision cell (second quadrupole). The collision gas was nitrogen with

30 eV collision energy. The product ions resulting from the collision were scanned over a range of *m/z* 10 to 1395 with a step size of 0.1 amu and a dwell time of 2 ms.

Sequence analysis

PSI-Blast (Altschul *et al*., 1997) and a homology based fold prediction program (Huynen *et al*., 1998) were used to predict the catalytic residues and the fold of AEH. The secondary structure elements of AEH and glutaryl acylase from *Bacillus laterosporus* (Aramori *et al*., 1991b) were predicted using the consensus of the following programs: PSIPred (Jones, 1999), Jpred (Cuff and Barton, 1999) and SAM-T99sec (Karplus *et al*., 1998).

RESULTS AND DISCUSSION

Expression of AEH in *E. coli* with C-terminal His_6-tag

To achieve a higher expression level and an easier purification of AEH than what was obtained with a previous construct (Polderman-Tijmes *et al*., 2002a) the *aehA* gene was cloned in pBAD/*Myc*-HisA (pBADAT), coupling both the *myc*-epitope and the His_6-tag C-terminally to the protein. The use of the arabinose promoter in the pBADAT plasmid resulted in an overproduction of 5 fold (1% of the total protein in cell-free extract) compared to the expression in the wild-type *A. turbidans* strain (Polderman-Tijmes *et al*., 2002a). Furthermore, with the resulting construct, the number of necessary purification steps was reduced from 4 to 2 by use of a Ni^{2+}- agarose column (Table 2). Two mg of more than 90% pure protein could be obtained from a 5-liter culture and was stable at 4 °C for at least 60 days. The attachment of the tag resulted in an increase of 2 kDa of the molecular mass of the protein, as is clearly visible on an SDS-PAGE gel (Fig. 1). To check if the properties of AEH had changed upon addition of the *myc*-epitope and the His_6-tag, the kinetic parameters of the purified enzyme for cephalexin hydrolysis were measured (Table 3) and compared to untagged recombinant protein (Polderman-Tijmes *et al*., 2002a). The K_M values of both proteins appeared to be similar (0.45 and 0.34 mM, respectively). The k_{cat} of the fusion protein is somewhat lower than for the untagged recombinant protein (347 s^{-1}), but the values are in the same order of magnitude. This indicates that proper folding of the recombinant protein occurs and shows that there is no dramatic influence of the additional C-terminal amino acids.

Conversion of p-NPGB by AEH

To check if the earlier observed inhibition by *p*-NPGB (Polderman-Tijmes *et al*., 2002a) was irreversible, the enzyme was preincubated with

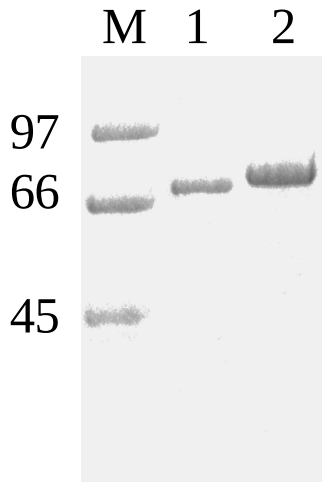


Figure 1. SDS-PAGE of AEH with and without C-terminal His_6-tag. The proteins were separated on 12.5% SDS-PAGE and stained with Coomassie brilliant blue. Lane M, molecular weight marker with the masses indicated at the left in kDa; Lane 1, AEH isolated from *E. coli* BL21(DE3)-pLysS(pETAT); Lane 2, AEH with the *Myc*-epitope and His_6-tag isolated from *E. coli* TOP10(pBADAT).

Table 2. Purification of C-terminal His_6-tagged AEH from *E. coli*.

Purification step	Total volume (ml)	Total protein (mg)	Total activity[a] (cexU)	Specific activity[a] (cexU/mg)	Purification (fold)	Recovery (%)
Cell-free extract	72	312	5551	17.8	1	100
Ni^{2+}-agarose	4.8	2.7	3587	1329	75	65
Gelfiltration	8.0	2	3344	1672	94	60

[a] Cephalexin synthesis

p-NPGB and then mixed with substrate solution. Upon dilution into the NIPGB solution the inactivated enzyme gradually reverted to the active form (Fig. 2A). After 20 min the enzyme recovered a major part of its activity, indicating that the inactivation by *p*-NPGB involves a reversible modification at the active site. To further test the conversion of *p*-NPGB, AEH was incubated with *p*-NPGB and the formation of *p*-NP was followed by stopped flow spectroscopy. The reaction with *p*-NPGB followed a biphasic time course (Fig. 2B), consisting of an initial burst of *p*-NP followed by a phase that corresponds to the steady state rate of hydrolysis. The formation of the acyl-enzyme intermediate is faster than its hydrolysis, resulting in an accumulation of the acyl-enzyme and a burst of *p*-NP, which is in agreement with what is expected for an active-site-directed covalent inhibitor. Subsequently the acyl-enzyme complex is slowly hydrolyzed with a k_{cat} of $1.3 \pm 0.6 \times 10^{-3}$ s^{-1} (α_2,144 kDa). The steady state rate of conversion of *p*-NPGB within the concentration range of 0.1 to 1 mM *p*-NPGB was constant (data not shown), indicating that the K_M for *p*-NPGB is lower than 0.1 mM. Therefore, the burst at 1 mM *p*-NPGB can directly be related to the number of active sites. The burst was measured in duplicate with two different enzyme concentrations and was found to correspond to 2.7 ± 0.7 µM released product with 1.32 µM enzyme and 1.1 ± 0.2 µM with 0.66 µM enzyme. In view of the subunit composition, this indicates that each subunit has one active site.

Table 3. Kinetic parameters of cephalexin hydrolysis for mutants of AEH.

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1}/mM)	Relative activity (%)
AEH-His_6	0.45 ± 0.06	274 ± 7	609	100
S205A	-	< 0.1 [#]	-	< 0.01
Y206A	4.0 ± 0.2	120 ± 2	30	44
D338A	0.35 ± 0.09	0.201 ± 0.004	0.6	0.07
H370A	0.43 ± 0.06	0.190 ± 0.002	0.4	0.07
R85A	0.18 ± 0.06	184 ± 6	1022	67
S156A	0.25 ± 0.08	132 ± 3	528	48
H610A*	0.9 ± 0.2	> 69 ± 2	> 77	> 25

* Partially purified, approximately 30% pure; [#] No conversion was observed, detection limit.

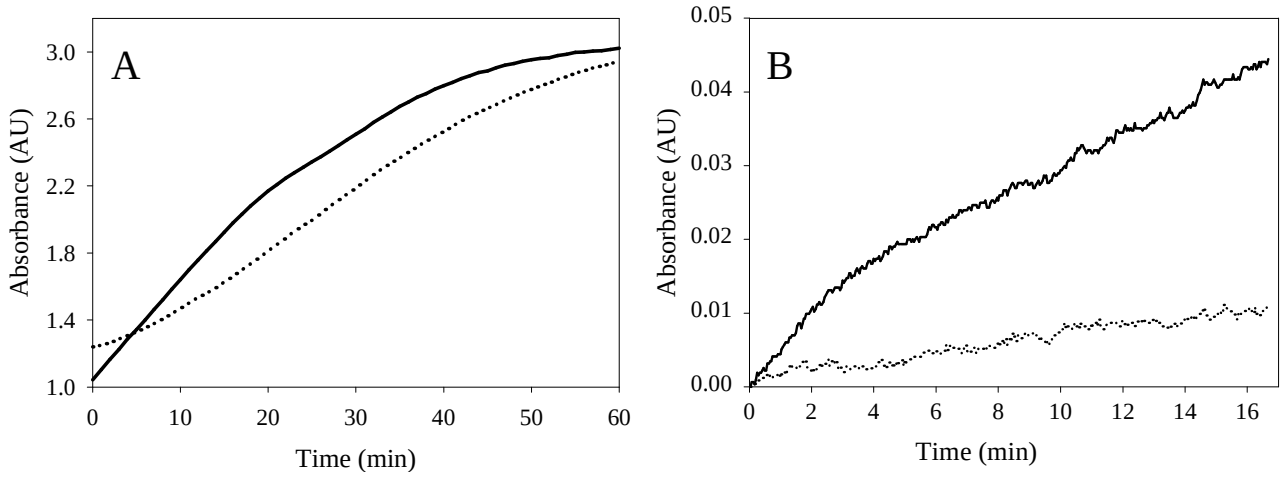


Figure 2. A. Reactivation of AEH after preincubation with *p*-NPGB. Solid line, untreated enzyme; dotted line, enzyme preincubated for 15 min with 1 mM *p*-NPGB in 1% DMF. The release of *p*-NP was followed. **B**, **Time course of reaction of AEH with of 1 mM *p*-NPGB.** Dotted line, chemical hydrolysis at 30 °C *p*-NPGB, solid line, conversion by 0.68 µM AEH.

Identification of the active site Ser-205 by labeling by p-NPGB

The slow conversion of the acyl-enzyme intermediate during reaction of *p*-NPGB made it possible to covalently label the enzyme (Fig. 3). AEH was incubated with excess *p*-NPGB and the covalent form was trapped by the addition of acid and subsequently fragmented with CNBr. Twenty peptide fragments in which the methionines had been modified to homoserine lactone were generated, varying in mass from 0.102 to 20.9 kDa. The elution pattern of the peptide mixture obtained from labeled AEH showed a few different peaks compared to the control (Fig. 4). These peaks were individually collected and analyzed by ES/MS. The peak indicated as control in the HPLC elution pattern (Fig. 4) corresponded to the fragment 562-GGYELPVSM-570

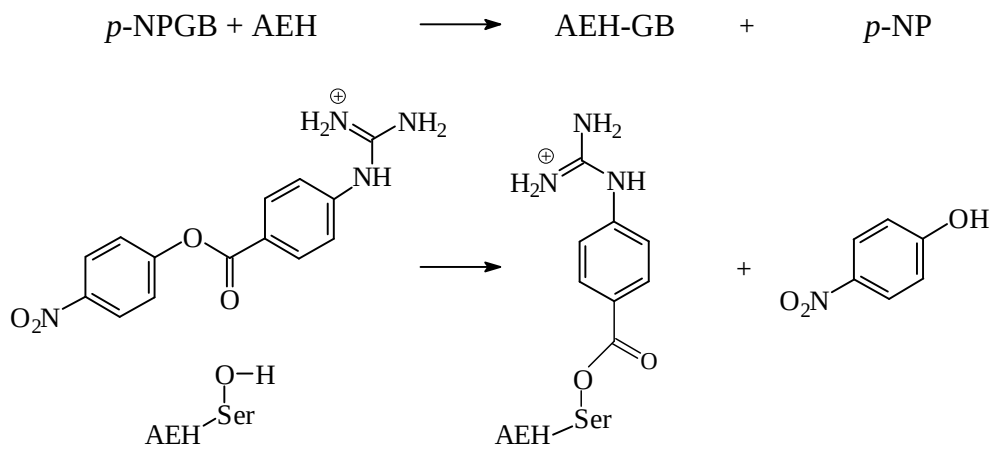


Figure 3. Reaction scheme showing the labeling of AEH by *p*-NPGB. The inhibitor *p*-NPGB reacts with the catalytic serine of AEH, resulting in *p*-NP and a labeled enzyme (AEH-GB).

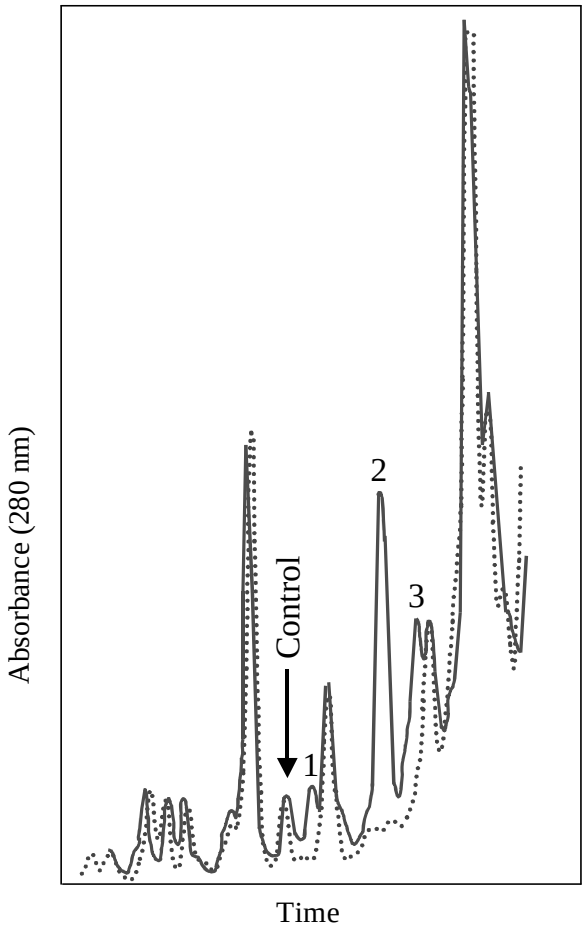


Figure 4. HPLC elution pattern of CNBr-peptide fragments of labeled (solid line) and unlabeled (dotted line) AEH. The peak indicated as control and peaks 1-3 were analyzed by mass spectrometry.

(903.4 Da), indicated by its singly, $(M + H)^+$, and doubly, $(M + 2H)^{2+}$, charged peak, *m/z* 904.4 and 452.6, respectively. This fragment had the same mass when isolated from unlabeled or *p*-NPGB-labeled protein (Fig. 5A and 5B). Peak 1 could not be assigned to an expected CNBr fragment and is likely the result of incomplete digestion. ES/MS analysis of peak 3 showed a mixture of peptides, of which the major component did not change upon labeling. The peptide eluting in peak 2 was identified as CNBr fragment 202-TGSSYEGFTVVM-213 (1228.6 Da) of which *m/z* 1229.5, $(M + H)^+$, and *m/z* 615.6, $(M + 2H)^{2+}$, were present in the ES/MS analysis of the unlabeled protein (Fig. 5C). When isolated from protein that was preincubated with *p*-NPGB, a peptide was found at this position with a mass of 1390 Da, indicated by the peaks with by *m/z* 1390.8, $(M + H)^+$, and *m/z* 696.0, $(M + 2H)^{2+}$ (Fig. 5D). This mass is in agreement with the fragment of 1228.6 Da plus the guanidino benzoate label (161 Da, Fig. 6A), indicating that the fragment that harbors the potential active site serine in the serine protease sequence motif is labeled by *p*-NPGB. The increase in absorbance of the peptide after labeling is in agreement with the attachment of an aromatic group. The presence of the $(M + H)^+$ ion at *m/z* 1229.5 in the spectra of the labeled peptide fragment is probably due to some fragmentation in the orifice skimmer region of the mass spectrometer, resulting in loss of the charged label.

To determine which serine (204 or 205) of the fragment 202-TGSSYEGFTVVM-213 was modified by *p*-NPGB, the labeled peptide was analyzed by ES/MS/MS using product ion scan to obtain the significant fragments. The product ion scan of the precursor ion *m/z* 1229.5, $(M + H)^+$, of the unlabeled peptide, displayed most of the possible b^+-fragments, together with the precursor ion itself (Fig. 6B/C). The product ion scan of the $(M + 2H)^{2+}$ ion at *m/z* 695.9 of the labeled peptide showed an increase in the masses by 161 Da of the b^+-fragments starting at b_4, compared to the unlabeled protein (Fig. 6B/C). The same increase in the mass was found for the only detectable y fragments, y_9^+ (hsl-Ser205) to y_{11}^+ (hsl-Gly203) of the labeled peptide compared to wild-type (data not shown). Both the b and complementing y fragments that were found are in agreement with the label positioned on Ser205, and exclude labeling at Ser204.

Active site topology and site-directed mutagenesis

An alignment of AEH with homologous proteins that were identified with BLAST (Altschul *et al.*, 1997) revealed a number of conserved residues in AEH (Fig. 7). To identify other putative catalytic residues, we searched for homology with proteins having a known structure by extended homology searches using PSI BLAST (Altschul *et al.*, 1997) and fold prediction (Huynen *et al.*, 1998). AEH shows 29% identity with cocaine esterase that has an α/β-hydrolase fold and a catalytic triad (Larsen *et al.*, 2001). The catalytic residues of this triad align with Ser205, Asp338 and His370 in AEH (Fig. 7). The N-terminal part (residue 67 to 374) of AEH showed 12% identity with proline iminopeptidase (34 kDa) from *Xanthomonas campestris* pv. *citri* (pdb 1AZW). Its catalytic domain also exhibits an α/β-hydrolase fold and is considered to be a suitable model for the catalytic domain of the prolyl oligopeptidase family (Medrano *et al.*, 1998) (Fig. 7). The catalytic Ser, Asp and His of this protein align with the same residues from AEH as indicated above. In the same region of AEH, 11% identity was found with prolyl aminopeptidase (36 kDa) from *Serratia marcescens* (pdb 1QTR). This enzyme has a similar crystal structure

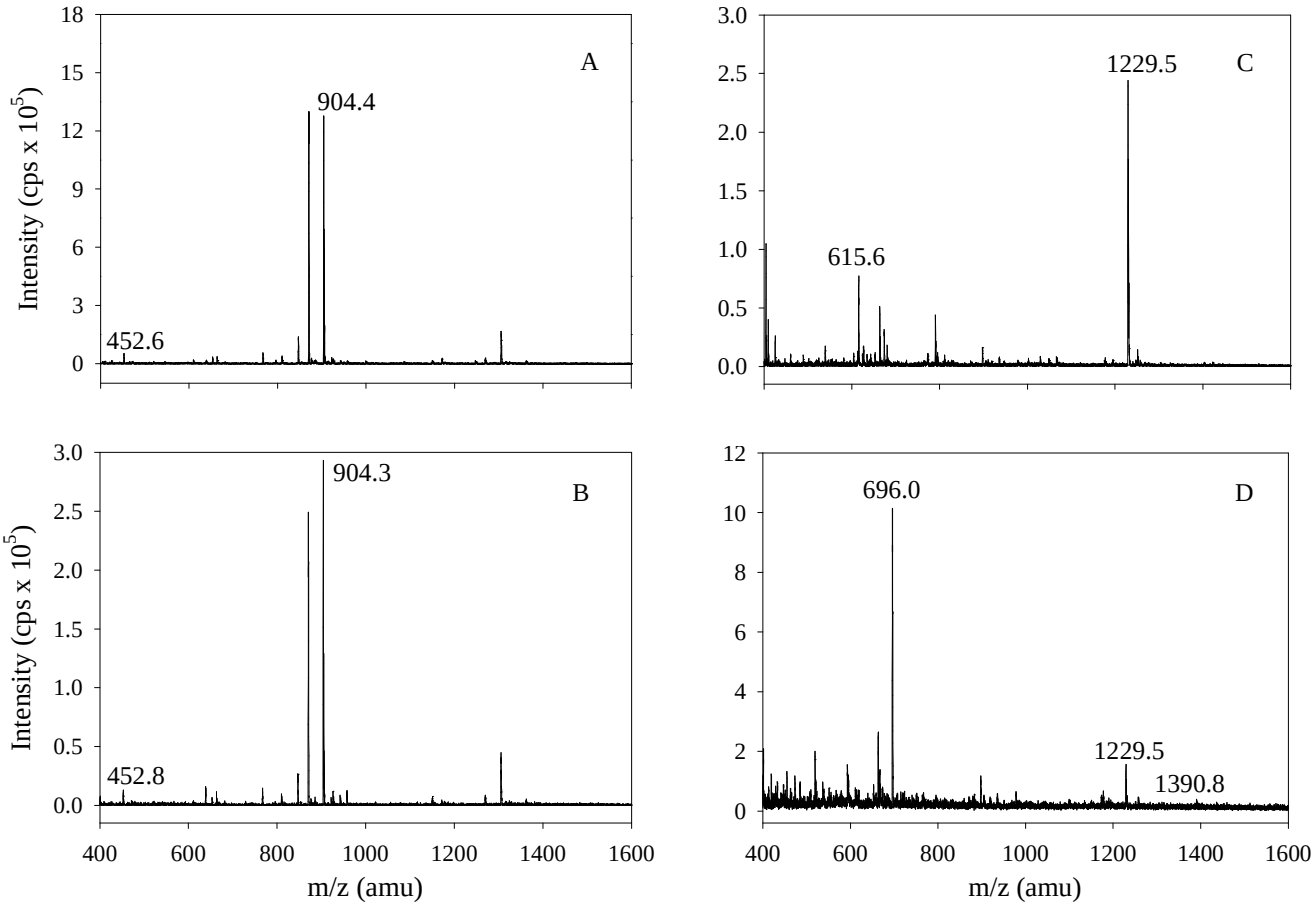
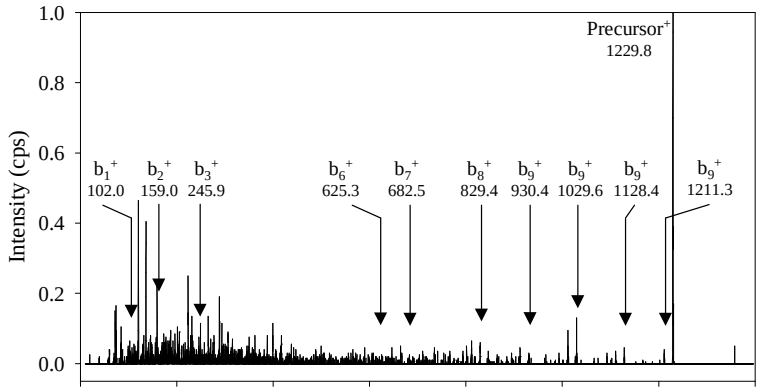


Figure 5. ES/MS-spectra of the CNBr peptide fragments generated from AEH after labeling with by *p*-NPGB. Shown are MS spectra of peptide Gly562-Met570 (control peptide, panels A and B), and of peptide Thr202-Met213 (panels C and D). The peptides were obtained from unlabeled enzyme (panels A and C) or from AEH preincubated with *p*-NPGB (panels B and D). Cps: counts per second; amu: atomic mass unit.

A

	202 Thr	Gly	204 Ser	205 Ser	Tyr	Glu	Gly	Phe	Thr	Val	Val	213 hsl
	b1	b2	b3	b4	b5	b6	b7	b8	b9	b10	b11	b12
b^+	**102.1**	**159.1**	**246.1**	333.1	496.2	**625.3**	**682.3**	**829.3**	**930.4**	**1029.5**	**1128.5**	**1211.6**
+ label:												
b^{+204}	102.1	**159.1**	407.2	**494.2**	**657.3**	**789.3**	**843.3**	**990.4**	**1091.4**	**1190.5**	**1289.6**	1372.6
b^{+205}	102.1	**159.1**	**246.1**	**494.2**	**657.3**	**789.3**	**843.3**	**990.4**	**1091.4**	**1190.5**	**1289.6**	1372.6

B



C

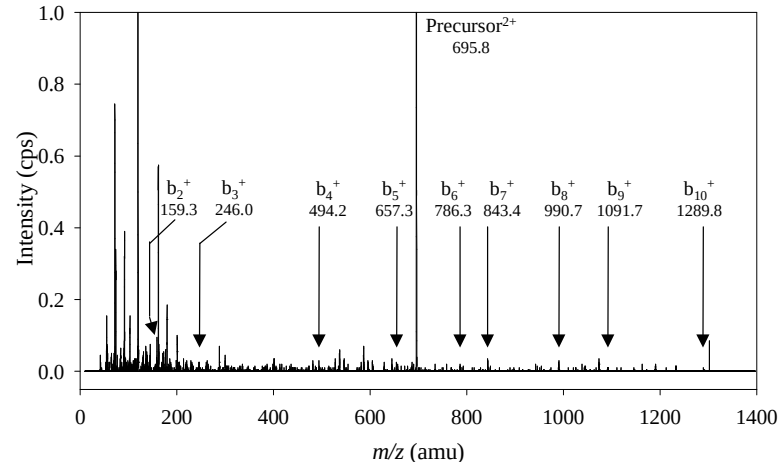


Figure 6. ES/tandem mass spectrometry analysis of peptide Thr202-Met213. (A) The peptide sequence and its calculated monoisotopic singly-charged masses for the product ions of type b of the unlabeled (b^+) and the peptide labeled either at position 204 (b^{+204}) or position 205 (b^{+205}). Met213 is modified to a homoserine lactone (hsl). The *m/z* values observed in the spectra are shown in bold. (B) The product ion scan spectrum of the precursor ion *m/z* 1229.5 obtained with peptide Thr202-Met213 from the unlabeled enzyme. (C) The product ion scan spectrum of the precursor ion *m/z* 695.9 obtained with the same peptide from the labeled enzyme.

(Yoshimoto *et al*., 1999) as the proline iminopeptidase from *X. campestris* and its catalytic triad residues align in the same way with AEH. In a smaller region (residue 94 to 370) 11% identity was found with a chloro- and bromoperoxidase (pdb 1A7U, 30.3 kDa (alignment shown in Fig. 7) and 1BRT, 30.2 kDa, respectively). The catalytic nucleophile and acid of these α/β-hydrolase fold enzymes (Hofmann *et al*., 1998) align with AEH at position 205 and 338, respectively. Prolyl oligopeptidase from porcine muscle (pdb 1QFM, 80.2 kDa) has a catalytic

Figure 7. Conserved regions of AEH and structure-based alignment with homologous α/β-hydrolase fold enzymes. Alignments were done using Clustal W 1.8 with blosum62 weight matrix and default parameters. Grey-shaded bold residues are identical and grey-shaded residues are similar among the proteins used for the alignment. The consensus of the secondary structure predicted for AEH, without its signal sequence, is shown. Residues located in a β-sheet are underlined and residues in a helix are underlined with a wave. Symbols: (▼), the catalytic residues; (-), residues involved in the stabilization of the oxyanion hole; (#) residues mutated to an alanine. The sheets are numbered and the helices are alphabetically labeled. Used abbreviations: Coc - Cocaine esterase from *Rhodococcus* sp., pdb. 1JU3; Pro - Proline iminopeptidase from *X. campestris*, pdb. 1AZW; Chl - Chloroperoxidase T from *Streptomyces aureofaciens*, pdb. 1A7U; Oli - Prolyl oligopeptidase from porcine muscle, pdb. 1QFM.

domain with an α/β-hydrolase fold structure and shows 13% identity with AEH (residue 71-268) and alignment of its active site serine with Ser205. Extending the alignment of chloroperoxidase and prolyl oligopeptidase with AEH manually on basis of the predicted structural elements resulted in the conservation of the other catalytic residues as well (Fig. 7).

Based on these alignments, a catalytic triad of Ser205, Asp338 and His370 is expected, which is supported by the identification of Ser205 as the catalytic nucleophile by the labeling experiments. To support this, Asp338 and His370, together with Ser205 as a control, were mutated to an alanine. Other conserved residues from AEH, specifically Arg85, Asp86, Tyr143, Ser156, Tyr206, Asp509, and His610 were also mutated to an alanine. All mutants were properly expressed in the pBAD vector, except for the mutants Asp86Ala, Tyr143Ala and Asp509Ala. Under the different growth conditions tested no sufficient expression of these mutants for purification could be achieved. Therefore, these residues were assigned an important structural role. Slight variations in expression levels were observed for the other mutants, but they were similar to wild-type AEH according to their behavior in the standard purification procedure. The effects of the mutations on the ability to hydrolyze cephalexin were determined (Table 3). Replacement of Ser205, Asp338 or His370 by an alanine reduced the activity drastically. These radical changes were not observed for the other purified mutants. The effects of the inactivating mutations on the secondary structure were evaluated with circular dichroism. The spectra obtained with the purified mutant and wild-type enzymes were superimposable (Fig. 8) and the calculated percentages of the secondary structure elements were essentially the same as calculated from the wild-type data. According to the data the wild-type enzyme had 25.4% α-helices, 43.2% β-sheets (beta-turns, antiparallel and parallel sheets) and 31.4% random coil. Therefore, from the CD-spectra we conclude that the inactivation caused by the mutations of Ser205, Asp338 or His370 did not result from drastic changes in the secondary structure of the enzyme.

The K_M for cephalexin was in the same order of magnitude as found for wild-type enzyme for all the active mutants, except for the Tyr206 mutant, of which the K_M increased significantly (Table 3). A possible role for this residue will be given below. Residues Ser156, His610 and R85 do not seem to play an important role in the hydrolysis of cephalexin. Based on the kinetic characterization and the CD spectra of the mutants we conclude that AEH is a serine hydrolase and contains a classical catalytic triad of Ser205, Asp338 and His370.

Structural analysis

The alignments and the conservation of the catalytic triad residues suggest an α/β-hydrolase fold for AEH. This should also be evident from the arrangement of the secondary structure elements (Fig. 7). Secondary structure predictions yielded 16 β -strands and 7 β helices (3 or more residues predicted as strand or helix), excluding

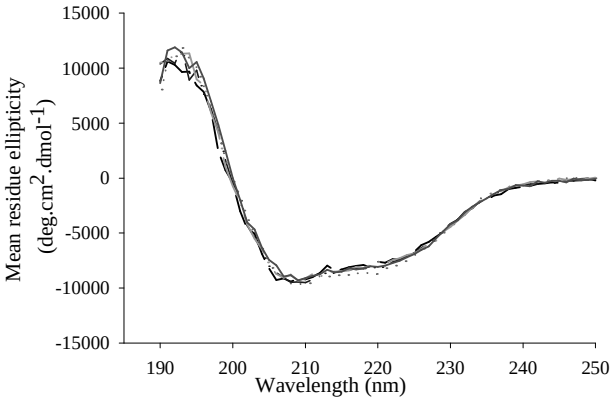


Figure 8. Superimposed circular dichroism spectra of wild-type AEH, the inactive mutants and the active mutant Tyr206Ala. The inactive mutants shown are Ser205Ala (- · · -), Asp338Ala (- · -) and His370Ala (· · ·). The CD spectrum of wild-type AEH (—) and the active mutant Tyr206Ala (---) is given.

the signal sequence. As found in α/β-hydrolase fold enzymes the catalytic residues in AEH are preceded by a strand and followed by a helix. Furthermore, the order of the structural elements in the N-terminal part of AEH is similar to that of the α/β-hydrolase fold enzymes, as is evident from a secondary structure driven alignment with chloroperoxidase, proline iminopeptidase and prolyl oligopeptidase (Fig. 7). A superimposition of the structural elements of AEH on the topology diagram of the α/β-hydrolase fold visualizes this very clearly (Fig. 9). An additional helix is predicted between strand 6 and 7, which is in agreement with the position of the additional domain in proline iminopeptidase. In other α/β-hydrolase fold enzymes additional helices are also found at this position and form a cap domain (Ollis *et al*., 1992). The positions of the structural elements relative to the catalytic residues further indicate that the catalytic domain of AEH, encompassing the N-terminal part of the protein sequence excluding the signal sequence (residues 41-416), has an α/β-hydrolase fold.

The function of the C-terminal part of AEH (417-667) which is predicted to harbor 8 or more β -strands, remains unclear. In cocaine esterase this domain adopts a jelly-roll like β-domain and is expected to be important for the arrangement of the overall tertairy structure (Larsen *et al*., 2001). A role in substrate specificity can also be expected, similar to the role of the N-terminally located β-propeller in prolyl oligopeptidase (Fülöp *et al*., 1998).

In α/β-hydrolase fold enzymes, the main chain NH group of the residue following the nucleophile is usually involved in the formation of the oxyanion hole, donating one of the two hydrogen bonds to the oxygen of the tetrahedral intermediate (Ollis *et al*., 1992). The second group stabilizing the oxyanion hole is usually located between β-strand 3 and helix A. In proline iminopeptidase Gly43 and Trp111 (Medrano *et al*., 1998) and in chloroperoxidase T, Phe32 and Ser99 (Hofmann *et al*., 1998) are suggested to constitute the oxyanion-binding site, both through their backbone NH groups. In oligopeptidase of porcine muscle and cocaine esterase the hydrogen bonds are provided by the main chain NH group of the residue following the catalytic serine and by the OH of a Tyr residue (Tyr44 and Tyr 473, respectively) (Fülöp *et al*., 1998; Larsen *et al*., 2001). Comparing the structural alignment and the

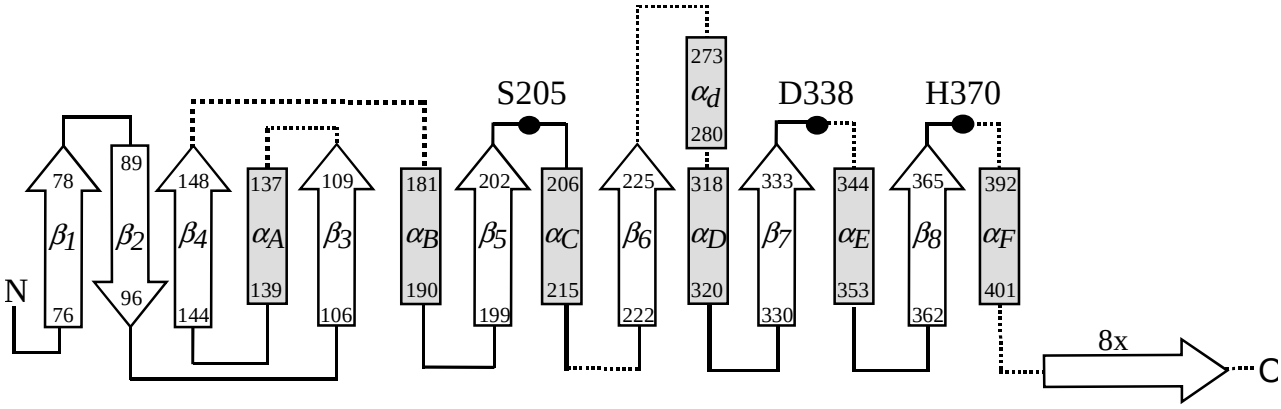


Figure 9. Predicted topology diagram of the N-terminal domain of AEH. The predicted structural elements were placed in a topology diagram for α/β-hydrolase fold enzymes resulting in a model for AEH. The arrows indicate strands and the boxes represent helices.

conserved residues the most likely candidate for the hydrogen donors in AEH are Tyr112, which aligns with the second hydrogen donor of cocaine esterase and prolyl oligopeptidase, and Tyr206 (Fig. 7). The removal of the hydroxyl group in the Tyr206Ala mutant did not abolish activity, indicating that also in AEH the backbone NH of this residue next to the catalytic serine is responsible for the stabilization of the oxyanion hole. Tyr112 is very likely the second hydrogen bond donor, probably through its hydroxyl group as found for cocaine esterase and prolyl oligopeptidase (Fülöp *et al*., 1998; Larsen *et al*., 2001).

In conclusion, the results presented in this paper indicate that AEH is an α/β-hydrolase fold enzyme and has a classical catalytic triad with Ser205 as the nucleophile. The enzyme has a small cap domain and an extensive C-terminal domain that is largely β-stranded. The enzyme is homologous to one other β-lactam antibiotic acylases, glutaryl 7-ACA acylase from *Bacillus laterosporus* (Aramori *et al*., 1991b), which is expected to have a similar structure. These results define a class of β-lactam antibiotic acylases that is clearly different from other known β-lactam antibiotic acylases that belong to the Ntn-hydrolase family.

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5

The genetic characterization and crystal structure of α-amino acid ester hydrolase from *Xanthomonas citri*

A model for a new class of β-lactam antibiotic acylases

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ABSTRACT

The α-amino acid ester hydrolases (AEHs) catalyze the hydrolysis and synthesis of β-lactam antibiotics. This chapter describes the cloning of the gene, the expression in *Escherichia coli* and the 1.9-Å resolution crystal structure of the AEH from *Xanthomonas citri* IFO 3835. The structure shows that the enzyme consists of three domains, (i) an α/β-hydrolase fold domain, (ii) an helical cap domain and (iii) a jellyroll β-domain. Structural homology was observed to the cocaine esterase from a *Rhodococcus* sp., indicating that both enzymes belong to the same class of serine hydrolases. The α/β hydrolase fold provides the framework for a classical catalytic triad, which is in agreement with the results from sequence analysis and inhibition studies for the AEH of *A. turbidans*. Docking of a β-lactam antibiotic in the active site of AEH explains the substrate specificity, including the necessity of the α-amino group in the substrate, and explains the low specificity toward the β-lactam nucleus. This it the first crystal structure of an AEH and as such provides a strong basis for rational engineering of this class of β-lactam acylases.

INTRODUCTION

β-Lactam antibiotics form a large family of widely applied antibacterials. Their successful career started with the discovery and production of penicillin G and was expanded with the development of semi-synthetic analogs in which the naturally occuring acyl group of β-lactam antibiotics like penicillin G and cephalosporin C is replaced by a synthetic acyl group. Initially, this was achieved by chemical means but at present enzymatic methods are preferred (Bruggink and Roy, 2001). A well-known enzyme used for these conversions is penicillin acylase (EC 3.5.1.11) from *Escherichia coli*. This enzyme is used both for the production of the β-lactam nuclei by cleaving off the side chain, for example from penicillin G or cephalosporin G, and for the coupling of new acyl groups to free β-lactam nuclei to obtain semi-synthetic β-lactam antibiotics (Bruggink and Roy, 2001). Unfortunately, penicillin acylase is strongly inhibited by the phenyl acetic acid that is generated next to the free β-lactam nucleus (6-aminopenicillanic acid, 6-APA) during cleavage of penicillin G. Thus, before the β-lactam nucleus can be used in a coupling reaction, any traces of phenyl acetic acid must be removed, necessitating an extra step in the production process. In addition, β-lactam nuclei are not very stable at the alkaline pH optimum of penicillin acylase. By contrast, α-amino acid ester hydrolases (AEHs) catalyze the hydrolysis and synthesis of esters and amides of α-amino acids also at low pH and are not inhibited by phenylacetic acid (Kato *et al.*, 1980b; Takahashi *et al.*, 1972). They can be used to acylate various β-lactam nuclei using an ester as acyl donor (Fig. 1), generating widely used antibiotics such as cephadroxil, cephalexin, ampicillin and amoxicillin. Other attractive properties of AEHs are their preference for esters and their stereospecificity toward the acyl donor (Blinkovsky and Markaryan, 1993; Fernandez-Lafuente *et al.*, 2001).

One of the first AEHs that was purified and characterized is the enzyme from

Xanthomonas citri (Hyun *et al*., 1993a; Hyun *et al*., 1993b; Kato, 1980; Kato *et al*., 1980a; Kato *et al*., 1980b; Rhee *et al*., 1980; Takahashi *et al*., 1972). This enzyme was found to be a homotetramer with subunits of 72 kDa (Kato *et al*., 1980a). Other known AEHs have similar subunit sizes (70-72 kDa) and are described to be dimers or tetramers (Kim and Byun, 1990; Polderman-Tijmes *et al*., 2002a; Ryu and Ryu, 1987). The recently cloned gene encoding the AEH from *Acetobacter turbidans* showed no coding sequence similarity to any known penicillin or cephalosporin acylase, except for the glutaryl 7-ACA acylase from *Bacillus laterosporus* (Aramori *et al*., 1991b). Additionally, no N-terminally located Thr, Ser or Cys, the hallmark of the Ntn-hydrolase superfamily to which penicillin acylase and other β-lactam acylases belong was found for the *A. turbidans* enzyme (Polderman-Tijmes *et al*., 2002a). Kinetic studies indicated the occurrence of an acyl-enzyme intermediate in the hydrolysis and synthesis of β-lactam antibiotics by the AEHs (Blinkovsky and Markaryan, 1993; Kato, 1980; Nam *et al*., 1985; Takahashi *et al*., 1974). Labeling, site-directed mutagenesis studies, and secondary structure predictions identified a catalytic triad of Ser, Asp and His within a putative α/β-hydrolase fold domain for the AEH of *A. turbidans* (Polderman-Tijmes *et al*., 2002b). Based on these observations, the AEHs are considered to form a new class of β-lactam acylases (Polderman-Tijmes *et al*., 2002b).

In this paper, we report the detailed characterization of the AEH from *X. citri*. The cloning and overexpression of its gene facilitated protein isolation and crystallization experiments, which resulted in an X-ray structure with a 1.9-Å resolution. The *X. citri* AEH structure is considered prototypical for the AEH-class of β-lactam acylases and together with the inhibition studies, an examination of the substrate range, and the kinetic analysis presented here, it provides insight in the structure-function relationship of the AEHs.

MATERIALS AND METHODS

Materials

Antibiotics and related substrates were provided by DSM Life Sciences (Delft, the Netherlands). D-2-Nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) was obtained from Syncom (Groningen, The Netherlands). D-2-Nitro-5-[(phenylacetyl)amino]benzoic acid (NIPAB), L-alanine *p*-nitroanilide (ANA) and *p*-nitro-phenylacetic acid were retrieved from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands).

The oligonucleotides for cloning of the AEH gene from *X. citri* (*aehX*) were provided by Eurosequence BV (Groningen, the Netherlands). Chemicals used in DNA manipulation procedures were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and used as recommended by the manufacturer. The DNA sequences were determined at the Department of Medical Biology of the University of Groningen.

Bacterial strains, plasmids and growth conditions

X. citri IFO 3835 was grown for 16 h at 28 °C in a 10-liter fermentor using the medium described by Takahashi *et al.* (1972) with the addition of 0.005% (w/v) $FeSO_4$. *Zymomonas mobilis* ATCC 31821 was grown on the ATCC medium 1341 at 30 °C. *E. coli* strains HB101

(Boyer and Roulland-Dussiox, 1969) and the methionine deficient strain *E. coli* B834(DE3) (Novagen, Inc. Madison, WI) were used for cloning derivatives of pEC (DSM Life Sciences). *E. coli* XL1-Blue MR (Stratagene, La Jolla, CA) was the host for a genomic library of *X. citri* in the cosmid pWE15 (ampicillin resistant, Stratagene). For production of selenomethionyl-incorporated protein, *E. coli* B834(DE3) carrying the construct expressing *aehX* (pXc, chloramphenicol resistant) was grown for 30 h at 30 °C on M9 medium (Sambrook *et al*., 1989) supplemented with a vitamin and spore solution (Janssen *et al*., 1984), D/L-selenomethionine (100 mg/l), chloramphenicol (34 mg/l) and iso-propyl-β-D-thiogalactopyranoside (0.4 mM). *E. coli* TOP10 (Invitrogen, Breda, the Netherlands) was used for cloning derivatives of pBAD/*Myc*-HisA (Invitrogen). For vector isolation the *E. coli* strains were grown at 30 °C on LB supplemented with chloramphenicol or ampicillin.

Isolation of α-amino acid ester hydrolase from *X. citri* and amino acid sequence determination

The α-amino acid ester hydrolase (AEH) from *X. citri* was purified by cation exchange, hydrophobic interaction chromatography and gel filtration as described for the corresponding enzyme from *A. turbidans* AEH (Polderman-Tijmes *et al*., 2002a). Briefly, the retained protein was eluted from CM Sepharose using a linear gradient of 0-1 M KCl at 0.45 M KCl. From the Resource Phenyl Sepharose column the *X. citri* AEH activity eluted at approximately 0.36 M $(NH_4)_2SO_4$ in a decreasing linear gradient from 1.5 M to 0 M $(NH_4)_2SO_4$. Finally, the enzyme was purified to SDS-PAGE homogeneity by gel filtration (Sephacryl S300, 1.6 × 65 cm, Amersham Pharmacia Biotech, Hertfordshire, United Kingdom). The purification was monitored by measuring cephalexin synthesis activities (Alkema *et al*., 2000) and NIPGB hydrolysis (Polderman-Tijmes *et al*., 2002a). One activity unit (U, in cexU or NIPGB U) is defined as the amount of enzyme needed to produce or hydrolyze 1 µmol of cephalexin or NIPGB per min, respectively. For sequence analysis of *X. citri* AEH, approximately 100 µg of protein was sliced from a SDS-PAGE gel and digested with trypsin. From the digest those peptides containing a tryptophan (high absorbance at 297 nm) were selected for sequencing. Eurosequence BV (Groningen, the Netherlands) carried out further preparation of the sample and determined the amino acid sequence by automated Edman degradation (model 477A, Applied Biosystems).

Preparation and screening of the *X. citri* genomic library

The gene encoding the AEH from *X. citri* (*aehX*) was identified in a genomic-DNA cosmid-library by Southern blotting. To this end, genomic DNA was isolated from *X. citri* and partially digested with *Sau*3A as described before (Polderman-Tijmes *et al*., 2002a). The conditions were optimized to obtain fragments of 30-45 kb. The fragments were ligated in cosmid pWE15 (ampicillin resistant) that had been digested with *Bam*HI and dephosphorylated with alkaline phosphatase. *In vitro* packaging and infection of *E. coli* XL1Blue MR was carried out according to the recommendations of the manufacturer of the DNA packaging kit (Roche Diagnostics). Part of the *aehX* gene was cloned by PCR amplification from chromosomal DNA using two primers based on the obtained internal amino acid sequences, pF, 5'-AAYCCNAGYGARGTNGAYCAYGC -3' and

pR, 5'-YTTRTGCCACCANGGNARYTGYTC-3' (Y is T or C; R is A or G; N is any base). The PCR product was isolated from gel (Qiaquick kit from Qiagen, GmbH, Hilden, Germany), cloned and sequenced. A gene probe for the *aehX* gene was made using matching primers based on the DNA sequence of the PCR fragment. The forward primer was 5'-ACCGATGCCTGGGACACC-3' (up-stream of pF) and the reverse primer was 5'-CAGGCCTGCGGCCTTGGC-3' (downstream of pR). These primers were used to amplify a 317-bp fragment with Taq polymerase using the PCR DIG probe synthesis mix from Roche Diagnostics. To obtain the whole gene, colony hybridization of the genomic cosmid library using the specific probe was carried out as described previously for the cloning of the *A. turbidans* AEH (Polderman-Tijmes *et al*., 2002a). The nucleotide sequence of the α-amino acid ester hydrolase from *X. citri* has been submitted to GenBank and assigned accession no. AY162325.

Cloning of *aehX* into an expression host

For expression of the *aehX* gene in *E. coli*, it was placed under control of a *tac* promoter in the pEC vector (Alkema *et al*., 2000), resulting in pXc. For cloning in the *Nde*I/*Hin*dIII site of pEC the gene was amplified with the primers, 5'-TCCGGAGTC*ATTA**ATG***CGCCGCCTTGCCAC-3' (*Asn*I site in italics, start codon in bold) and a reverse primer, 5'-ACCGGTGCC*AAGCTT***TCA**-ACGTTACCGGCAG-3' (*Hin*dIII site in italics, stop codon in bold). After denaturation of the DNA (pWE15 (*aehX*)) amplification was performed in 30 cycles of 30 s at 94 °C, 1 min at 58 °C and 1 min and 30 s at 72 °C. Product and vector were subjected to restriction and subsequently ligated. The ligation mixture was used to transform *E. coli* HB101.

Cloning and expression of the gene encoding the AEH from *Z. mobilis*

The gene encoding the AEH from *Z. mobilis* ATCC 31821 (*aehZ*, GenBank accession no. AF124757, protein ID AAD29644) was cloned in the *Nde*I and *Hin*dIII site of pEC resulting in pZM. For this, the gene was amplified from the freeze-dried cells supplied by ATCC with the forward primer, pfz, 5'-CAGGGAGGG*CAT**ATG***TCTCGATTAAAGCTC-3' (*Nde*I site is shown in italics and start codon in bold) and the reverse primer, prz, 5'-TTTATTCTC*AAGCTT***TTA**TGGGATAACCGG CAA-3' (*Hin*dIII site is shown in italics and the stop codon in bold). The gene was also cloned in a modified pBAD/*Myc*-HisA vector. In this vector, the tree *Nde*I sites present in the original pBAD-vector were removed by site-directed mutagenesis and the multiple cloning *Nco*I site was changed into an *Nde*I site. The *aehZ* gene was amplified from pZm with the primers pfz and przhis, 5'-TTTATTCTC*AAGCTT*TGGGATAACCGGCAA-3' (*Hin*dIII site is shown in italics), and after restriction cloned in the *Nde*I and *Hin*dIII sites of the modified vector, resulting in pBADZm. All the constructs were confirmed by sequence analysis.

To obtain an active enzyme extract, *E. coli* Top10 (pBADZm) was grown at room temperature in the presence of ampicillin (Amp, 50 mg/l) and 0.0001% (w/v) arabinose. From a 2-liter culture the cells were harvested by centrifugation and resuspended in 50 mM Na-phosphate, pH 6.2. A cell extract was made using a French press and subsequent centrifugation for 15 min at 10,000 × g. The supernatant was subjected to ultra-centrifugation (1 h at 240,000 × g) and the pellet was homogenized in Na-phosphate, pH 6.2. Due to the presence of ampicillinase encoded by pBAD only the K_M for

7-amino-cephalosporanic acid derivatives could be determined for the AEH from *Z. mobilis*.

Enzyme assays and determination of kinetic constants

AEH activities were routinely measured by determining the rate of hydrolysis of activated side chains or β-lactam antibiotics with purified AEH or AEH-containing cell-free extracts at room temperature and at pH 7.0 (50 mM Na-phosphate buffer) unless stated otherwise. The hydrolysis and synthesis products were analyzed by HPLC (Polderman-Tijmes *et al.*, 2002a). For the determination of Michaelis Menten K_M and k_{cat} values, enzyme preparations were incubated with varying concentrations of substrates. Cephalexin hydrolysis was measured in the range from 0.95-15 mM; for ampicillin the range was 0.5-15 mM; for D-phenylglycine methylester 4-250 mM; for D-4-hydroxyphenylglycine methylester 0-30 mM; for amoxicillin 0.5-15 mM; and for cefatrizine 0.3-15 mM. The conversion of cephadroxil, D-phenylacetic acid methyl ester, and penicillin G was measured at 10 mM and D-phenylglycine amide at 50 mM. The conversion of the chromogenic substrates NIPAB (0-20 mM, $\Delta\varepsilon_{405nm}$ = 9.09 $mM^{-1}.cm^{-1}$), NIPGB (0-20 mM, $\Delta\varepsilon_{405nm}$ = 13 $mM^{-1}.cm^{-1}$) and nitrophenyl acetate (at 2 mM, $\Delta\varepsilon_{405nm}$ = 13 $mM^{-1}.cm^{-1}$) was measured in a spectrophotometer.

To determine the effect of inhibitors, the enzyme (5-10 nM, α_2, 140 kDa) was preincubated for 5 min with the inhibitors (4 mM) at 30 °C. The remaining initial hydrolysis activity (less than 10% conversion) of the enzyme on D-PGM (50 mM in 50 mM Na-phosphate, pH 6.2) was measured. Stock solutions (0.1 M) of the inhibitors were made in 50 mM Na-phosphate, pH 7.0 at 0.1 M, except for *p*-phenylmethyl sulfonyl fluoride, which was dissolved in methanol. 2,4-Dinitrobenzenesulfenyl chloride was used at 1 mM with 200 nM enzyme (final concentrations). The effects of the solvents were measured separately and when necessary used to correct the effect of the inhibitors.

Isolation of recombinant selenomethionyl-incorporated *X. citri* AEH from *E. coli*

Selenomethionine-AEH was purified from *E. coli* B834(DE3)(pXc) cells grown in the presence of 100 mg/l DL-selenomethionine. The selenomethionyl-incorporated enzyme was purified as described above with the addition of 5 mM dithiothreitol to all buffers to prevent oxidation of the selenium. The recombinant protein eluted at a higher concentration $(NH_4)_2SO_4$ (0.5 M) from the hydrophobic interaction column than found for the wild-type enzyme (see above). The enzyme was concentrated to 5 mg/ml in 20 mM Na-cacodylate buffer, pH 6.5 by ultrafiltration (YM30, Amicon).

Crystallization

Protein crystals were grown essentially as described earlier (Barends *et al.*, 2003b). Briefly, 1.5 µl of concentrated *X. citri* AEH was mixed with an equal volume of 12-15% PEG 8000 in 0.1 M cacodylate, pH 6.5 and equilibrated against 500 µl of this precipitant solution. Prior to freezing, crystals were briefly soaked in 15% PEG 8000 in 0.1 M MES, pH 6.5, to remove the arsenic-containing cacodylate, as arsenic has spectral properties comparable to those of selenium that interfere with MAD data collection around the selenium wavelength. Crystals were cryoprotected by soaking for a few seconds in 25% glycerol, 15% PEG 8000 in 0.1 M MES, pH 6.5.

Data collection, structure determination and model building

The incorporation of selenomethionine allowed the determination of the structure factor phases by the method of a multi-wavelength anomalous dispersion (MAD). Datasets of both native and selenomethionine-labeled *X. citri* AEH crystals were processed and refined with several software programs and additional programs were used to dock ampicillin in the active site (details have been published by Barends *et al.* (2003b)). The resulting 1.9-Å model contains 2452 amino acids (four chains of 613 residues each), 1806 water molecules, four calcium ions and nine glycerol molecules.

RESULTS AND DISCUSSION

Purification and characterization of the AEH from *X. citri*

The α-amino acid ester hydrolase from *X. citri* was purified by ion-exchange, hydrophobic interaction and gel filtration column chromatography (Table 1). Using gel filtration, the native enzyme was found to be present mainly as a dimer, with subunits of 72 kDa (Fig. 1). The enzyme rapidly hydrolyzed substrates with an α-amino group, like D-PGM and cephalexin. The k_{cat}–values for these substrates (Table 2) are 3-fold and 10-fold lower, respectively, than those reported by Kato *et al.* (1980b), calculated from methanol detection by GC, but are in good agreement with those found for the AEH from a *Xanthomonas* sp., determined by HPLC analysis (Blinkovsky and Markaryan, 1993). The enzyme was hardly active with substrates having no α-amino group such as penicillin G, *p*-nitrophenyl acetate and phenylacetic acid methyl ester (Table 2). This confirms the need for an α-amino group in the substrate as indicated in literature (Kato *et al*., 1980b). The presence of a hydroxyl group on the *para*-position of the phenylglycine side chains in both the ester and antibiotic derivative (HPGM and amoxicillin, respectively) resulted in a drastic decrease of the specificity (k_{cat}/K_M) compared to the analogue without it (PGM and ampicillin, respectively). The combination of the *p*-hydroxyphenylglycine with a 7-aminodes-acetoxycephalosporanic acid (7-ADCA) nucleus (cephadroxil) was not accepted at all. This became evident from both hydrolysis and synthesis experiments. Surprisingly, the enzyme was able to couple *p*-hydroxylphenylglycine to other

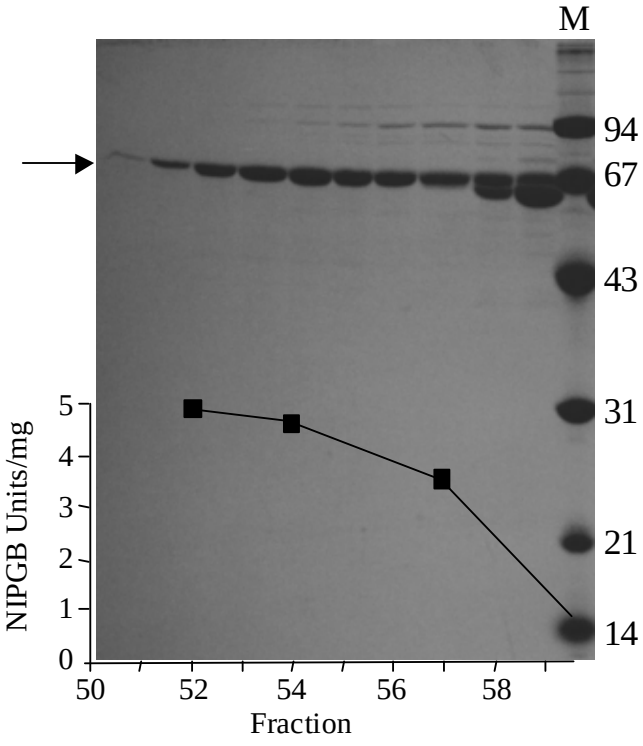


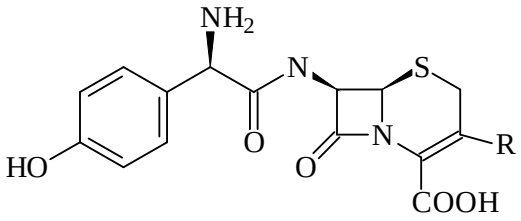
Figure 1. SDS-PAGE of *X. citri* AEH. The enzyme was purified as described in Materials and Methods. Shown are the fractions 50-59 of the gel-filtration step and their specific NIPGB activities. A molecular mass marker was loaded in the lane labeled M and the masses (in kilo Daltons) are indicated at the right of the gel. The arrow indicates the AEH.

Table 1. Purification of the α-amino acid ester hydrolase from *X. citri* IFO 3835.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (cexU)	Sp. activity (cexU/mg)	Purification (fold)	Recovery (%)
CVE	420	1890	2457	1.3	1	100
CM-sepharose	134	48	1394	29	22	57
Resource phenyl	25	2.62	178	68	52	7
Sephacryl S300	1.1	0.1	28	288	222	1.1

cephalosporin nuclei, like 7-amino-cephalosporanic acid (7-ACA), 7-amino-3-chloro-cephalosporanic acid (7-ACCA) and 7-aminodesacetyl cephalosporanic acid (7-ADAC). Additionally, hydrolysis was observed for the β-lactam antibiotic cefatrizine, which is *p*-hydroxyphenylglycine coupled to 7-amino-3-((1H-1,2,3-triazol-4-ylthio)methyl)-cephalosporanic acid (7-ATTC). These nuclei have different substituents on the third position of the dihydrothiazine ring (Fig. 2). Unlike the substituent of 7-ADCA (CH_3), these groups are polarisable. However, in combination with other acyl groups 7-ADCA is accepted by the enzyme, like with cephalexin (phenylglycine coupled to 7-ADCA) which is synthesized and hydrolyzed with high activity. The binding of an acyl donor with a hydroxyl group seems to influence the binding of the nucleus by the enzyme. These observations indicate a complicated and highly interactive substrate-binding site.

A chromogenic compound that is hydrolyzed by AEHs is 2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB), an amino-substituted derivative of the well-known chromogenic substrate for *E. coli* penicillin acylase, 2-nitro-5-[(phenylacetyl)-amino]-benzoic acid (NIPAB). However, the k_{cat} of NIPGB (Table 2, (Polderman-Tijmes *et al*., 2002a)) is quite low, which makes its use time and enzyme consuming. In search for a better chromogenic substrate, alanine-4-nitroanilide (ANA) was tested. The enzyme indeed showed a 295-fold higher k_{cat} for this substrate and a higher specificity in terms of k_{cat}/K_M than for NIPGB (Table 1). ANA is a very suitable substrate to monitor the purification of an AEH, although one should take



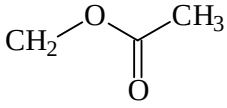
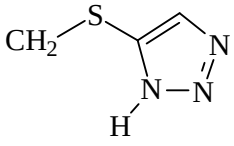
Nucleus	R	Substrate
7-ACA		+
7-ADCA	CH_3	-
7-ACCA	Cl	+
7-ADAC	CH_2OH	+
7-ATTC		+

Figure 2. The acceptance of *p*-hydroxyphenyl cephalosporins by the AEH from *X. citri*. The different substituents on the 3-position of the cephalosporanic acid nucleus are shown.

Table 2. Kinetic parameters of *X. citri* AEH.

Substrate	K_M (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)
D-phenylglycine amide	-	1.6	
D-phenylglycine methyl ester	90	1860	21
D-hydroxy-phenylglycine methyl ester	-	-	9[b]
Ampicillin	1.2	58	48
Amoxicillin	-	-	2[b]
Cephalexin	1.8	160	89
Cephadroxil	-	n.c.	
Cefatrizine	< 1	2.3	> 2.3
D-phenylacetic acid methyl ester	-	n.c.	
Penicillin G	-	n.c	
NIPGB	0.07	0.4	5.7
ANA	1.0	82	82
Nitrophenylacetate	-	0.1	

n.c., no conversion detected; -, not determined; [a], calculations with 140 kDa dimer; [b], determined from initial slope of Michaelis-Menten curve.

in account that other amidases are expected to have a high specificity for this substrate as well. NIPGB can be useful as a chromogenic reference substrate for kinetic analysis of non-chromogenic substrates, since its k_{cat}/K_M is lower than that of most other substrates (Table 2) (Alkema *et al*., 1999).

Identification of essential residues

To identify the amino acids crucial for the catalytic activity of AEH of *X. citri*, we measured the effect on the hydrolytic activity of several chemical reagents known to react with specific amino acid side chains (Table 3). Compounds that react with active site histidine residues, L-1-4'-tosylamino-2-phenylethyl chloromethyl ketone, diethylpyrocarbonate and 1-fluoro-2,4-dinitro-benzene, reduced the activity significantly. A large effect was also seen with 4-(2-aminoethyl)-benzene-sulfenyl fluoride (Pefabloc SC), a compound that inhibits serine proteases. The AEH activity was not inhibited by the penicillin acylase inhibitor *p*-phenylmethyl sulfonyl fluoride, see also Nam *et al.* (1985), which can be explained by the absence of an amino group. The observed inhibition by 2,4-dinitrobenzenesulfenyl chloride and *N*-bromossucinimide points towards an important tryptophan residue. Concluding, at least a serine, a histidine and a tryptophan residue are important for catalytic activity.

Cloning of the gene (*aehX*) encoding the AEH of *X. citri*

To allow amino acid sequencing, the purified AEH was subjected to digestion by trypsine since the N-terminal sequence appeared to be blocked. From the digest two tryptophan-rich fragments were isolated and their amino acid sequences determined, which yielded Ala-Ala-Gly-Leu-Glu-Gln-Leu-Pro-Trp-Trp-His-Lys and Gly-Pro-Leu-Asn-Pro-Ser-Glu-Val-Asp-His-Ala-Thr-Asp-Ala-Trp-Asp-Thr-Ile-Asp-Trp. Based on the alignment of the internal fragments with homologous proteins from the database, the direction of the primers needed to amplify part of the *aehX* gene could be determined. Two degenerated primers were designed (pF and pR) and used to amplify an internal part of the sequence. As expected from the alignment with homologous proteins, a PCR product of 0.4 kb was obtained, sequenced and used to design matching primers to generate a DIG-labeled

Figure 3 (opposite page). Sequence alignment of AEHs and 7-ACA glutaryl acylase from *B. laterosporus* and cocaine esterase from *Rhodococcus* sp. Abbreviations: Xc, *X. citri*; Xf, *X. fastidiosa*; At, *A. turbidans*; Zm, *Z. mobilis*; Bl, *B. laterosporus*; and Rsp, *Rhodococcus* sp. The individual domains as determined from the *X. citri* AEH structure are indicated above the sequence. Symbols: *, Active site residues; -, the residues forming the oxyanion hole; +, residues involved in binding the amino moiety; x, the residues constituting the calcium binding motif; #, residue stabilizing the carboxylate cluster involved in binding of the amino moiety; □, residue likely stacking the ring of the β-lactam nucleus; ●, residue likely stacking the phenyl ring of the side chain (stacking with side chain of Asp-208 is also predicted but not indicated here); ■, residues restricting the phenyl binding pocket; s, residues forming salt bridges between monomers; and c, cysteine residues forming a disulfide bridge. For clarity the sequences from *X. axonopodis* pv. *citri* and *X. campestris* were not included in the alignment. The alignment was made using Clustal W 1.8 with blosum62 weight matrix and default parameters and colored with BoxShade (available on the world-wide-web). Black shaded residues are identical and grey shaded ones are similar.

probe. This probe was used to screen a 99.9% complete genomic pWE15-cosmid library (2,300 colonies) of *X. citri* DNA in *E. coli*. Out of the 768 colonies screened one hybridized with the probe, for which no AEH activity could be detected. Of the insert the gene encoding the AEH from *X. citri* (*aehX*) and 1 kb upstream and 100 bp downstream of it was sequenced. The first 621 bp coded for the C-terminal part of a putative protein with 64% sequence similarity to a glutamate-5-semialdehyde dehydrogenase from *Meiothermus ruber* (protein ID no. O86053). This enzyme is involved in the proline biosynthesis. Separated by 353 bp from this putative gene we detected the start of the *aehX* gene with a possible ribosomal binding site at position -7.

The AEH family of β-lactam acylases

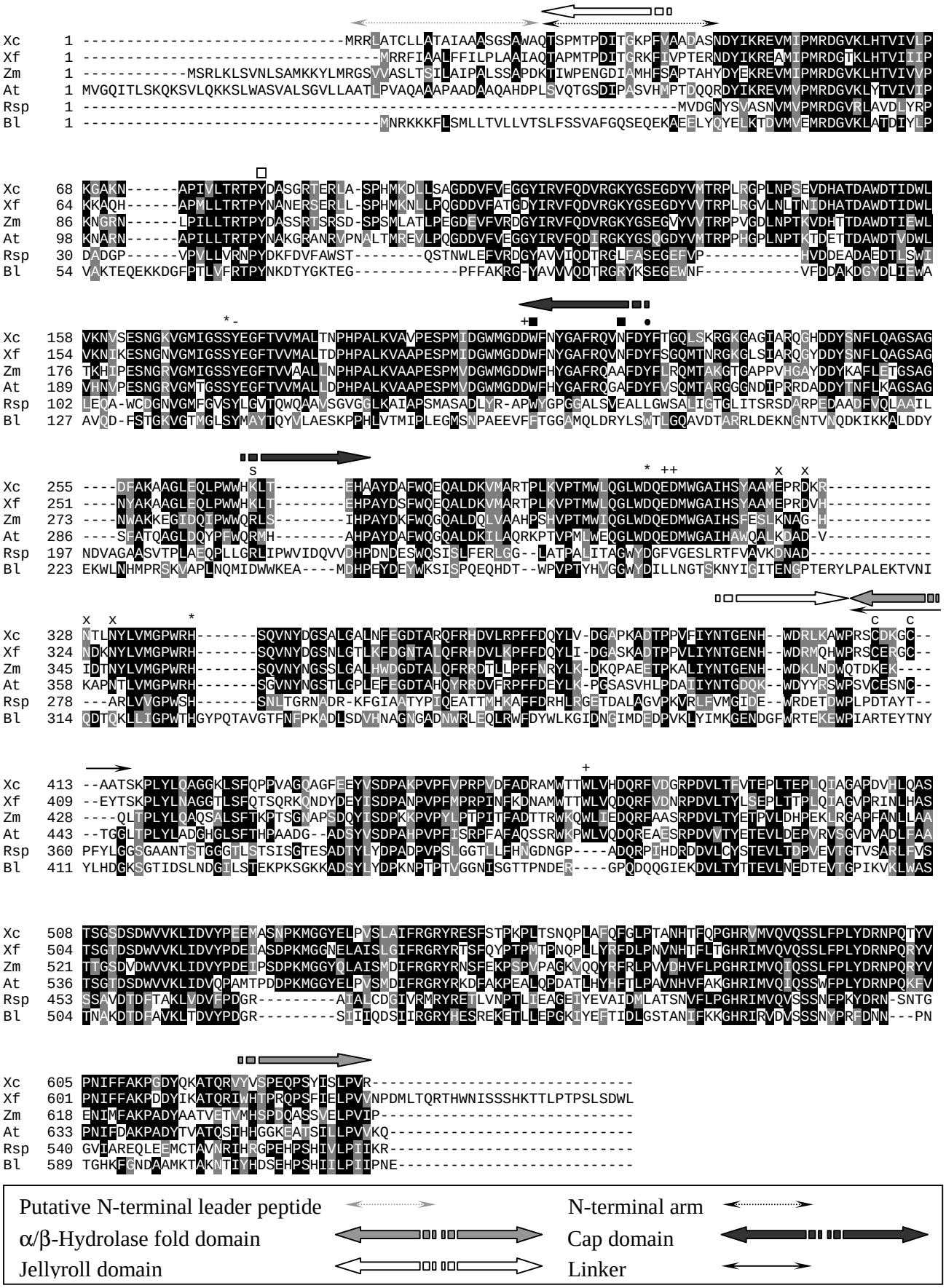
The gene encoding the α-amino acid ester hydrolase from *X. citri* encodes a polypeptide of 637 amino acids with a calculated molecular weight of 70,915. Sequence analysis revealed a possible N-terminal signal sequence of 22 residues with its cleavage site after the consensus pattern for the periplasmic signal peptidase I (AXA, 20-AWA-22) (Fig. 3) (Nakai, 2000; Nielsen *et al.*, 1997). The presence of the signal sequence suggests a periplasmic localization for AEH, which was not further explored in this study. Possible enzymatic cyclization of Gln23 after the cleavage of the signal sequence and subsequent removal of the formed pyroglutamate by enzymatic action of a pyrrolidone-carboxylate peptidase (Awade *et al.*, 1994) is in agreement with the N-terminus of the crystal structure which could be solved from Thr-24 onwards.

A BLAST search (Altschul *et al.*, 1997) with the deduced amino acid sequence revealed an

Table 3. Effect of inhibitors on hydrolysis of PGM by *X. citri* AEH.

Inhibitor	Residual activity (%)
None	100
Phenylmethylsulfonyl fluoride	90
Iodoacetate	95
4-(2-aminoethyl) benzenesulfonyl fluoride	13
TCK	46
N-Bromosuccinimide	0
Chloroacetone	78
N-Acetylimidazole	90
Phenylglyoxal	89
2,3- Butanedione	83
Diethylpyrocarbonate	75
1-Fluoro-2,4-dinitrobenzene	77
Cyanogen bromide	84
β-Mercaptoethanol	86
2,4-Dinitrophenylsulfenyl chloride	65

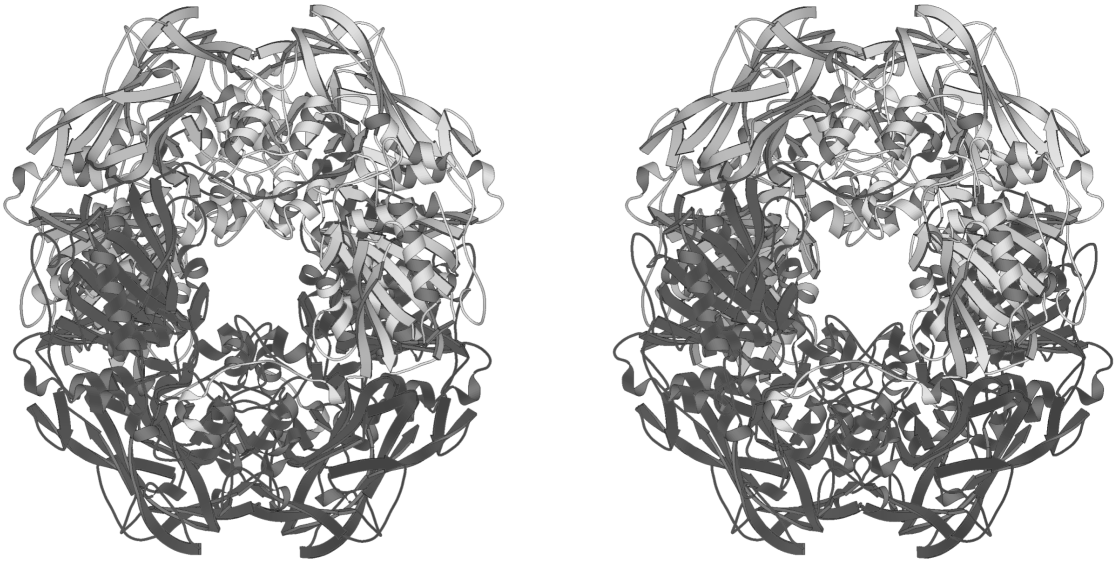
TCK: L-1-4'-Tosylamino-2-phenylethyl chloromethyl ketone


Putative N-terminal leader peptide
N-terminal arm
α/β-Hydrolase fold domain
Cap domain
Jellyroll domain
Linker

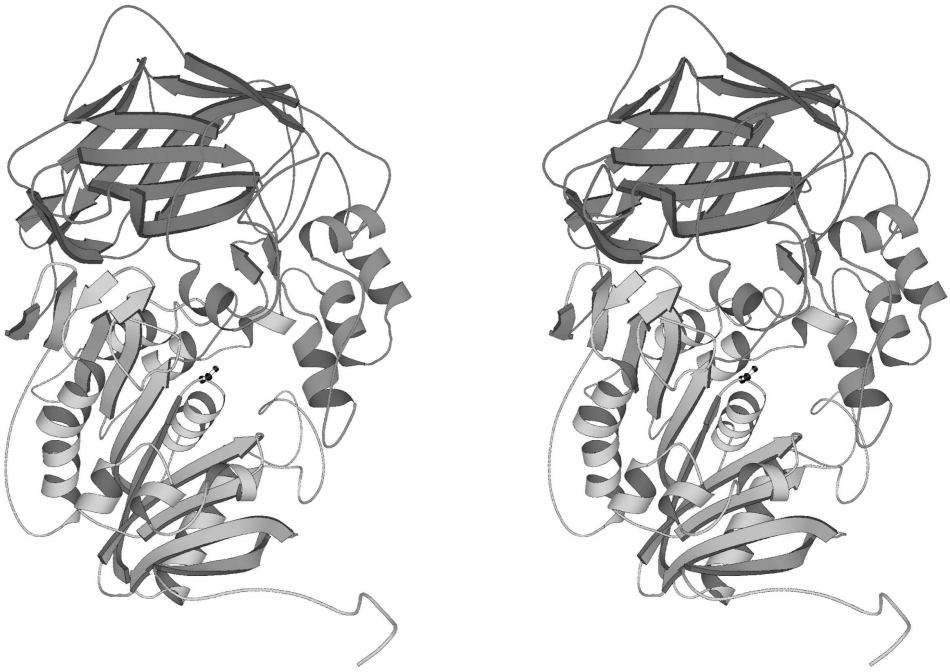
identical sequence in the databases annotated as a glutaryl 7-ACA acylase from *Xanthomonas axonopodis* pv. *citri* strain 306 (protein ID no. AAM37193) (da Silva *et al*., 2002). Other proteins with high identities (93, 78 and 62%) (Altschul *et al*., 1997)) to the AEH from *X. citri* are, respectively, the putative glutaryl acylases from *X. campestris* pv. *campestris* strain ATCC 33913 (protein ID no. AAM41516) (da Silva *et al*., 2002), *Xylella fastidiosa* (protein ID no. AAF83839) (Simpson *et al*., 2000), *Zymomonas mobilis* (protein ID no. AAD29644) and the α-amino acid ester hydrolase from *A. turbidans* (protein ID no. AF439262) (Polderman-Tijmes *et al*., 2002a). Lower sequence identities (< 28%) were found with the glutaryl 7-ACA acylase from *Bacillus laterosporus* and a cocaine esterase from *Rhodococcus* sp. (CocE, protein ID no. AAF42807 (Bresler *et al*., 2000) of which the structure has been published (Larsen *et al*., 2001).

The catalytic triad residues of the AEH from *A. turbidans*, Ser205, Asp338 and His370, were conserved in the AEH from *X. citri*, which is in agreement with the results from the inhibition studies. The high percentage of identity of the putative glutaryl 7-ACA acylases from *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris*, *X. fastidiosa* and *Z. mobilis* with the AEHs of *X. citri* and *A. turbidans* (> 60%) together with the conservation of the catalytic triad residues (Fig. 3) suggest that these proteins have amino ester hydrolase activity rather than glutaryl acylase activity. To confirm this, the gene encoding the putative glutaryl acylase from *Z. mobilis* was cloned and expressed in *E. coli* and its substrate range was explored. As expected, the enzyme was able to synthesize cephalexin from D-PGM and 7-ADCA. An initial synthesis over hydrolysis ratio of 1.9 ± 0.7 and a maximum product accumulation level of 5.5 ± 1.5 mM were measured, which is similar to the values found for the AEH from *A. turbidans* (*Chapter 6*). Since hydrolysis was observed for NIPGB, D-PGM and cephalexin but not for their α-amino group lacking analogous NIPAB, D-phenylacetyl methyl ester (D-PAM) and cephalosporin, it is concluded that the enzyme from *Z. mobilis* needs an α-amino group in the substrate for activity as well. In agreement with this, no hydrolysis of the chromogenic analog of glutaryl 7-ACA (glutaryl *p*-NA) was observed. The K_M values determined for cephalexin and D-phenylglycine methyl ester (PGM) were 2.0 ± 0.3 mM and 5.6 ± 0.5 mM, respectively. As found for the AEH from *X. citri* (Table 2), the AEH from *Z. mobilis* did not show activity for substrates with a *para*-hydroxyl moiety on the side group, especially not when attached to a 7-ADCA group, this in contrast to the enzyme from *A. turbidans* (Polderman-Tijmes *et al*., 2002a).

No sequence similarity of the AEH from *X. citri* to any known β-lactam acylases other than the glutaryl acylase from *B. laterosporus* was found, supporting the hypothesis that the AEHs together with the glutaryl acylase constitute a new class of β-lactam antibiotic acylases. The AEHs from *Pseudomonas melanogenum* (α_2, 72 kDa, (Kim and Byun, 1990)), *Xanthomonas rubrilineans* (α_4, 70-72 kDa, (Krest'ianova *et al*., 1990)) and *Achromobacter* B-402-2 NRRL B-5393 (Fujii *et al*., 1976), probably also belong to this class of β-lactam acylases. The presence of a homologous *aeh* gene was confirmed by a PCR with the primers pF and pR on whole cells of *X. rubrilineans* and *Achromobacter*, which yielded a product of 0.4 kb, as was found for *X. citri*.



A



B

Figure 4. Stereo representations of the *X. citri* AEH. A) Stereo representation of the *X. citri* AEH tetramer. In the picture we are looking through the large entrance into the central cavity. The monomers are alternately colored black or grey. **B**) Stereo representation of the *X. citri* AEH monomer. Each domain is individually colored, the N-terminal arm is depicted in the lower right-hand corner, the α/β-hydrolase fold is depicted in light grey (under left corner), the cap domain in dark grey (right side) and the jelly roll in black (top). The catalytic Ser-174 is shown in ball-and-stick representation.

Quaternary structure

The AEH of *X. citri* was overproduced in selenomethionine-labeled form and crystallized as described before (Barends *et al.*, 2003a). From the collected crystallographic data a structure was obtained with a resolution of 1.9 Å. Details of the crystallographic methods and the obtained quaternary structure have been published by Barends *et al.*, (2003b). In this chapter we will discuss only the general features of the quaternary structure of the enzyme.

The crystals revealed a tetrameric arrangement for the *X. citri* AEH, which is in agreement with gel filtration and ultracentrifugation studies of the *X. citri* AEH by Kato *et al.* (Kato *et al.*, 1980a). The four monomers form an approximate tetrahedron of ~100 Å (Fig. 4A) and enclose a large water-filled space with two large entrances. All four active sites are on the inside of the tetramer, facing the water-filled space, and are accessible only through these entrances.

The N-terminus of each molecule is observed from Thr24 onwards. From Thr24, the polypeptide forms a 40 Å long "arm" (residues 24-44) that lies on the surface of an adjacent monomer. This monomer, in turn, places its N-terminal arm onto the first monomer. Thus, the tetramer is composed of two dimers of which each monomer donates its N-terminus to the other molecule of the same dimer. The large entrances to the central cavity lie between these two arms. The monomers are mainly held together by hydrogen bonds, involving both main chain and side chain atoms. Only 4 salt bridges are present between the monomers, one for each arm-monomer interaction. This salt bridge is formed between the side chains of Asp30 of one monomer, and Lys270 of the other. These residues are conserved in the AEHs from the *Xanthomonas* strains, *X. fastidiosa, Z. mobilis* and *A. turbidans,* although Lys270 is an arginine in the AEHs from *A. turbidans* and *Z. mobilis*. Apart from this, the overall sequence homology with other AEHs in the arm region is much lower than in the rest of the protein (Fig. 3).

Monomer structure

The monomer (Fig. 4B) is composed of an N-terminal α/β-hydrolase fold (Ollis *et al.*, 1992) with large insertions, and a C-terminal domain that consists largely of β-strands with a jellyroll topology, with extra elements of secondary structure in the crossover loops. The α/β-hydrolase fold domain consists of a central, mostly parallel β-sheet of 10 β-strands, flanked on either side by α-helices (Fig. 4B and 5). The second strand runs antiparallel to the others. At the end of the fifth strand, a very tight turn into a helix contains Ser174, which is thus observed in the common position for the nucleophile in α/β-hydrolase fold enzymes (Nardini *et al.*, 1999; Ollis *et al.*, 1992).

The C-terminal domain adopts a jellyroll fold, which is connected to the α/β-hydrolase domain *via* a small linker, that is tightened into a compact structure by a disulphide bond between Cys408 and Cys412. These residues, are conserved in the AEH from *A. turbidans* and the putative AEHs from the *Xanthomonas* and *Xylella* species, but not in the one from *Z. mobilis* or the glutaryl acylase from *B. laterosporus* or CocE from *Rhodococcus* sp.

The fold of the monomer resembles that of the cocaine esterase from *Rhodococcus* sp. of which the structure was previously published (Larsen *et al.*, 2001). Whereas the α/β-hydrolase

and jellyroll domains are similar, differences are observed in the cap domain and the insertions in the jellyroll domain. The cap domain of CocE is larger, extending into the region where the entrance to the tetramer is found in *Xanthomonas citri* AEH. Arranging four CocE molecules in a way similar to the AEH tetramer shows that the entrances to the central cavity would be severely blocked by the larger cap domains of CocE. The structure of the *Lactococcus lactis* X-prolyl dipeptidyl aminopeptidase PepX, which was recently published, exhibits the same three-domain fold and organization as AEH and CocE , apart from an additional N-terminal domain involved in oligomerization (Rigolet *et al.*, 2002), like the N-terminal arms in AEH.

On the surface of the protein, on the outside of the tetramer and opposite the active site, a metal ion is bound by the side chains of Glu322 (by both carboxylate oxygen atoms), Asp325, Asn328 (through its Oδ) and the main chain carbonyl oxygen of Asn331. The ion was modelled as a calcium ion (Barends *et al.*, 2003b). This was unexpected since no divalent metal dependency was observed for *X. citri* or for any other known AEH by incubation with ethylenediaminetetraacetic acid (EDTA) (Blinkovsky and Markaryan, 1993; Kato *et al.*, 1980b; Takahashi *et al.*, 1974). Since the calcium seems strongly bound to the enzyme, stronger calcium chelating agents, such as EGTA, might be needed to remove the calcium. Sequence alignment of the proteins from *X. citri, A. turbidans, X. fastidiosa, B. laterosporus, Z. mobilis* and the cocaine esterase of a *Rhodococcus* sp. shows this motif to be unique to the *Xanthomonas* and *Xylella* AEHs (Fig. 3).

Active site structure

The catalytic Ser174 is found on the "nucleophilic elbow" between strand β5 and helix αC. Its position in this narrow turn places it near the N-terminal end of the 10-residue long helix C, which can stabilize negative charges through its helix dipole (Ollis *et al.*, 1992). Ser174 is roughly in the middle of a distinct active site pocket. It is in close contact with His340, which in turn is hydrogen bonded to Asp307. These residues constitute a catalytic triad (Fig. 5) and are found in the canonical positions in the sequence (Nardini *et al.*, 1999).

In α/β-hydrolases, the backbone amide of the residue directly following the catalytic nucleophile stabilizes the ionic intermediate by forming part of an oxyanion hole. In the case of *X. citri* AEH, a water molecule is observed close to the backbone NH of Tyr175 (Fig. 6), in the position expected for the oxyanion. An additional contribution to the stabilization of the oxyanion could be made by the side chain hydroxyl group of Tyr82, which forms a hydrogen bond with the water molecule.

The architecture of the oxyanion hole (Fig. 6) is identical to that observed in CocE and PepX. Apart from these enzymes, the only other enzyme known to use a tyrosine side chain in oxyanion stabilization is prolyl oligopeptidase. Is has been noted that due to the lower pK_a of tyrosine side chains, they could be more capable of oxyanion stabilization than the backbone or side chain amides used for this purpose in other enzymes (Fülop *et al.*, 1998; Szeltner *et al.*, 2000; Turner *et al.*, 2002). Furthermore, enzymes in which a side chain contributes to the oxyanion hole are considered ideal enzymological model systems

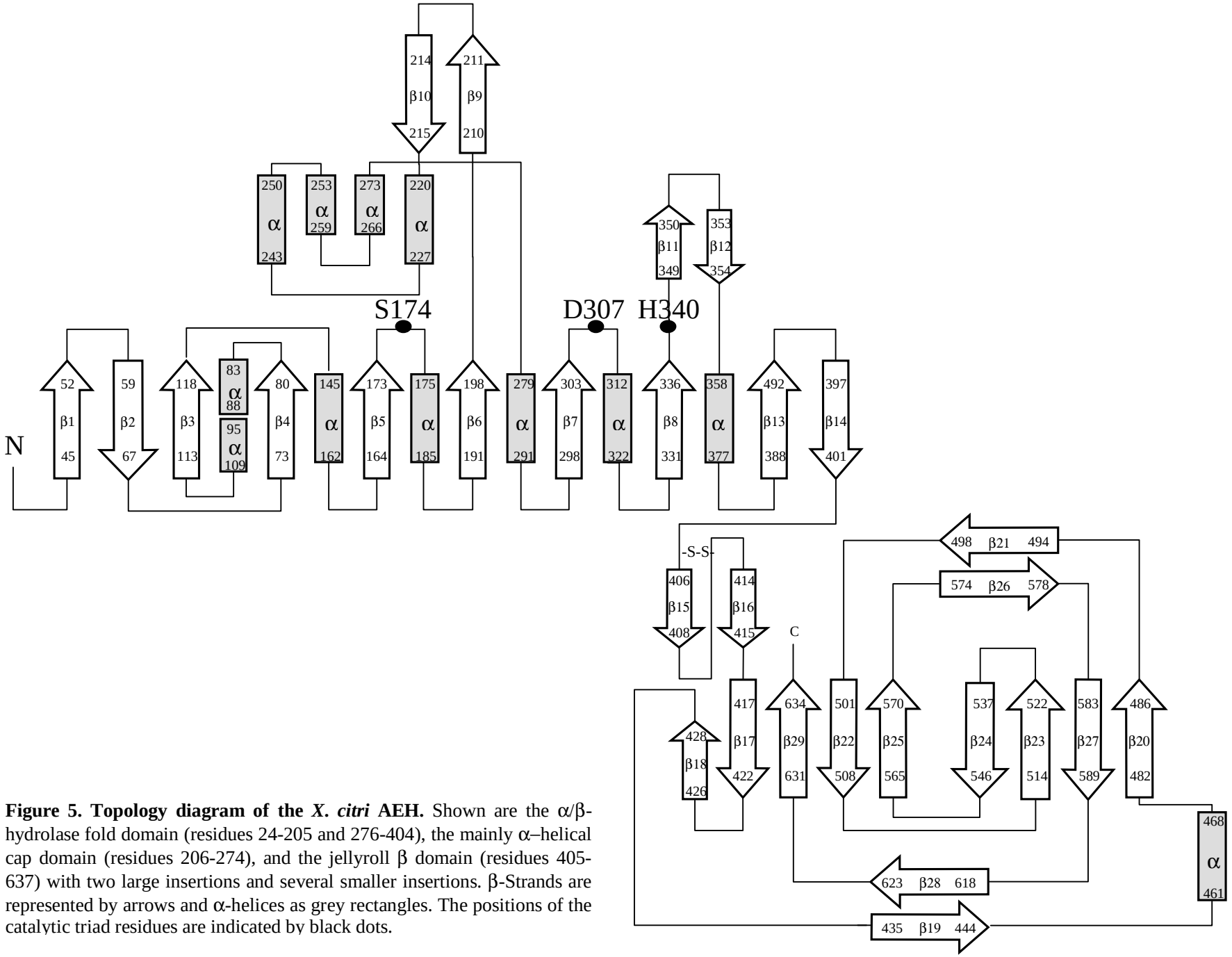


Figure 5. Topology diagram of the *X. citri* AEH. Shown are the α/β-hydrolase fold domain (residues 24-205 and 276-404), the mainly α–helical cap domain (residues 206-274), and the jellyroll β domain (residues 405-637) with two large insertions and several smaller insertions. β-Strands are represented by arrows and α-helices as grey rectangles. The positions of the catalytic triad residues are indicated by black dots.

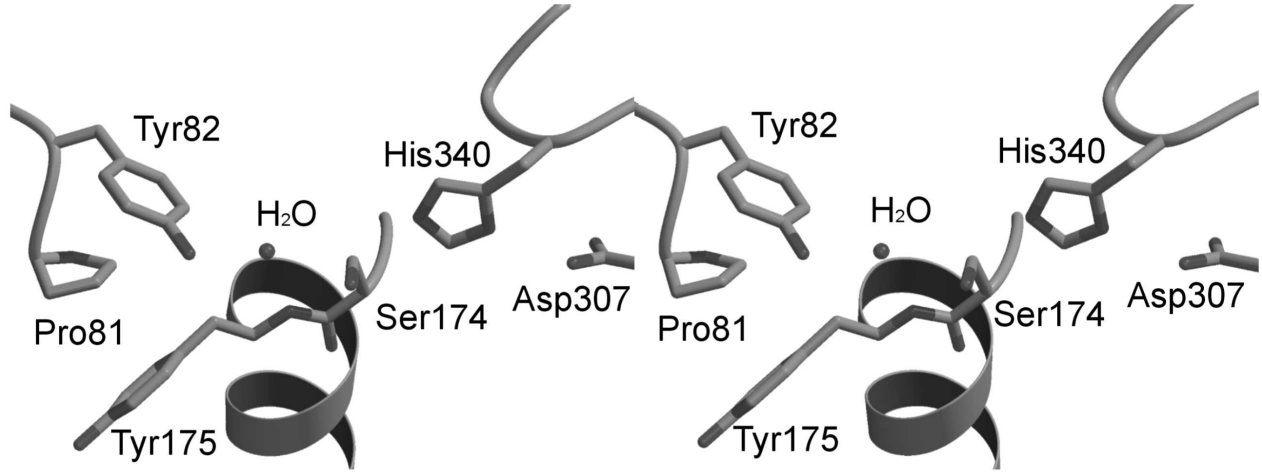


Figure 6. Stereo representation of the active site of the AEH from *X. citri*. The residues involved in binding and catalysis are shown in ball-and-stick representation.

because the oxyanion hole can be easily modified by site-directed mutagenesis (Szeltner *et al*., 2002).

Next to the catalytic histidine, a cluster of carboxylate groups protrudes into the active site (Fig. 6). This cluster is formed by Asp208, Glu309 and Asp310. These residues are conserved amongst the proteins from the proposed family of AEHs, but are not present in the *B. laterosporus* glutaryl acylase or the *Rhodococcus* sp. cocaine esterase. Consequently, this cluster of acidic residues may be important for recognizing the α-amino group of the substrate, which is crucial for activity (Table 2). This would also be in agreement with the preference of the enzyme for the positively charged form of the amino-containing substrates (Blinkovsky and Markaryan, 1993; Kato *et al*., 1980b).

The proximity of the side chains of Asp208, Glu309 and Asp310 in the active site would lead to an energetically unfavorable clustering of negatively charged atoms requiring stabilization. One way in which the enzyme stabilizes the clustering of negative charge is through a hydrogen bond of the Oε of Glu309 with the Nε of Trp465, which is conserved in the AEHs of the *Xanthomonas* strains, *X. fastidiosa, A. turbidans* and *Z. mobilis.* The importance of a tryptophan residue for the structural integrity is corroborated by the reduction in activity of the enzyme (65% residual activity) in the presence of 2,4-dinitrobenzenesulfenyl chloride, which reacts with tryptophan residues. Together with the high sequence homology, the conservation of the residues forming the carboxylate cluster in the putative acylases from *X. fastidiosa* and *Z. mobilis* identifies these proteins as AEHs, in contrast with their previous annotations as 7-ACA glutaryl acylases. On the other hand, the absence of these residues in the *B. laterosporus* sequence makes it unlikely that the latter protein is an AEH.

Modeling of substrate in the active site

To visualize how the cluster of Asp208, Glu309 and Asp310 could recognize the α-amino group of the substrate, an ampicillin molecule was manually docked into the active site based on the positions of the catalytic serine, the oxyanion hole

and the negatively charged cluster (Fig. 7). The amide bond of ampicillin was placed close to the nucleophilic Ser174, with the amide oxygen in the oxyanion hole, making hydrogen bonds to the backbone amide of Tyr175 and the phenolic OH of Tyr82. The α-amino group could then be positioned to make electrostatic interactions with the cluster of carboxylates formed by Asp208, Glu309 and Asp310. Aromatic ring stacking interactions occur between the aromatic ring of phenylglycine and Tyr222. Furthermore, the model predicts a stacking interaction between the phenylglycine ring and the side chain of Asp208. The side chains of Met200, Trp209, and Asp219 further delimit the pocket binding the phenyl ring. These residues belong to the cap domain, which shows a large degree of structural and functional variation within the α/β-hydrolase family (Nardini *et al*., 1999). In CocE, the cap domain binds the acyl group of the ester, which is consistent with our model. Since AEHs differ in their selectivity towards various antibiotics with substituted phenyl rings, differences in sequence would be expected in the regions just described.

The model predicts few interactions with the β-lactam nucleus, although stacking interactions with Tyr82 seem likely. In addition to this, some polar residues extend into the space where the β-lactam is located. The relative paucity of interactions with the β-lactam nucleus is consistent with the fact that both penicillin- and cephalosporin-derived nuclei can bind.

CONCLUSIONS

The β-lactams are still the most widely used antibiotics today, with an annual production of thousands of tons. Increasingly, chemical processes for antibiotic production are being replaced by more sustainable enzymatic conversions. The α-amino acid ester hydrolases (AEHs) show great potential for application in such processes. To understand the catalytic features of *X. citri* AEH, the gene was sequenced and the structure of the protein was solved. The tetrameric AEH of *X. citri* protein was composed of monomers that display a three-domain fold consisting of an α/β-hydrolase fold domain, a cap domain and a C-terminal domain with a jellyroll fold. Within the active site, a canonical Ser-His-Asp catalytic triad is observed but an interesting cluster of acidic residues close to the catalytic histidine sets the enzyme apart from other serine esterases. Given the remarkable specificity for

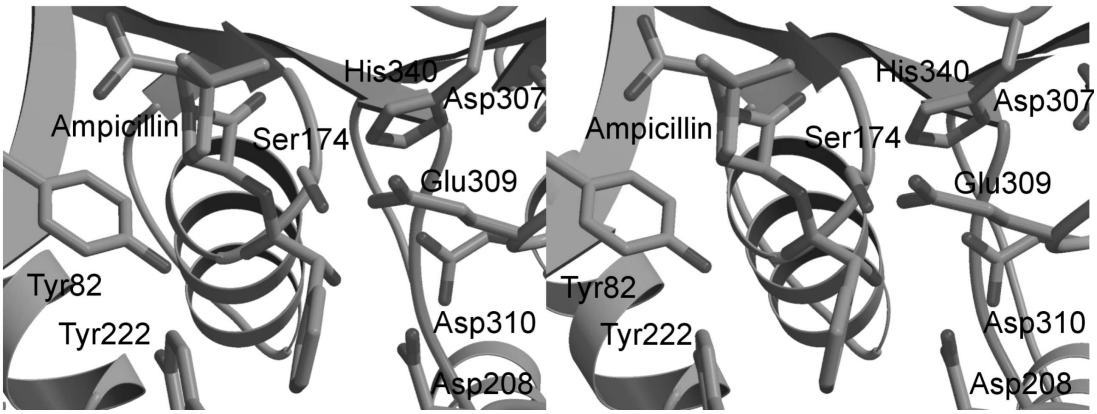


Figure 7. Stereo representation of ampicillin docked in the active site of *X. citri* AEH.

substrates with a charged ammonium group, it is likely that this "carboxylate cluster" is responsible for recognition and binding of this group, as illustrated by molecular modeling. The conservation of this cluster furthermore allows the definition of an AEH class at the genetic level, distinguishing the AEHs from structurally related esterases and peptidases.

Acknowledgements

Nanne Kamerbeek is acknowledged for providing the modified pBAD/*Myc*-HisA vector.

6

Enhanced β-lactam antibiotic synthesis by mutation of Tyr206 in the α-amino acid ester hydrolase from *Acetobacter turbidans*

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In preparation

ABSTRACT

The α-amino acid ester hydrolases (AEHs) can couple a phenylglycine group to a β-lactam nucleus, a reaction that is of great potential for the preparation of semi-synthetic antibiotics. The AEH from *Acetobacter turbidans* is a serine hydrolase of which the catalytic domain has an α/β-hydrolase fold that forms the scaffold for a classical catalytic triad composed of Ser205, His370 and Asp338. The oxyanion binding site is formed by the main chain amide of Tyr206 and the side chain hydroxyl group of Tyr112. To modify the catalytic properties of the enzyme, we mutated residue Tyr206 to seven amino acids with divergent properties. The mutant enzyme Tyr206Asn showed improved synthesis of both cephalexin and ampicillin.

INTRODUCTION

The α-amino acid ester hydrolases (AEHs) can transfer the acyl group of esterified α-amino acids to a nucleophile, which can either be water (hydrolysis) or an amide (aminolysis, Fig. 1). These enzymes are interesting from a biocatalytic point of view since they can be used to synthesize α-amino-substituted semi-synthetic β-lactam antibiotics from a synthetic α-amino acid ester and a β-lactam nucleophile such as 6-amino-penicillanic acid (6-APA, obtained from the fermentatively produced penicillin G). Recently, two α-amino acid ester hydrolases have been characterized in detail and with this a new class of β-lactam acylases was disclosed (Barends *et al.*, 2003b; Polderman-Tijmes *et al.*, 2002b).

The AEHs are serine hydrolases with a classical catalytic triad of serine, aspartate and histidine, as demonstrated by labeling and site-directed mutagenesis (Polderman-Tijmes *et al.*, 2002b). During the catalytic cycle the serine (Ser205 in *A. turbidans* AEH) becomes acylated, after which this covalent intermediate is cleaved by water or, in a synthesis reaction, by an incoming nucleophilic β-lactam compound (Fig. 1). Secondary structure alignments predicted that the catalytic domain would have an α/β-hydrolase fold, which was confirmed by the crystal structure of the AEH from *X. citri* (Barends *et al.*, 2003b). The presence of an α/β-hydrolase fold domain distinguishes the AEHs and the related glutaryl 7-ACA acylase of *Bacillus laterosporus* (Aramori *et al.*, 1991b) from the other known β-lactam acylases, which belong to the N-terminal nucleophile (Ntn) hydrolase superfamily (Duggleby *et al.*, 1995; Kim *et al.*, 2000; Suresh *et al.*, 1999). Each AEH subunit (approximately 70-72 kDa) consists of three domains: an α/β-hydrolase fold, a cap domain, and a jellyroll domain. A cluster of carboxylate groups involving one aspartate from the cap domain and another aspartate and a glutamate from the α/β-hydrolase fold domain were proposed to determine the absolute requirement for an α-amino group in the substrate. The jellyroll is thought to be important for the stability of the tertiary structure and is expected to influence the specificity for the α-amino group as well (Barends *et al.*, 2003b).

Analysis of the structure of the AEH from *X. citri* and sequence alignments of the AEHs with known α/β-hydrolase fold enzymes indicated that residues Tyr206 located next to the nucleophilic serine, and residue Tyr112 of the AEH from

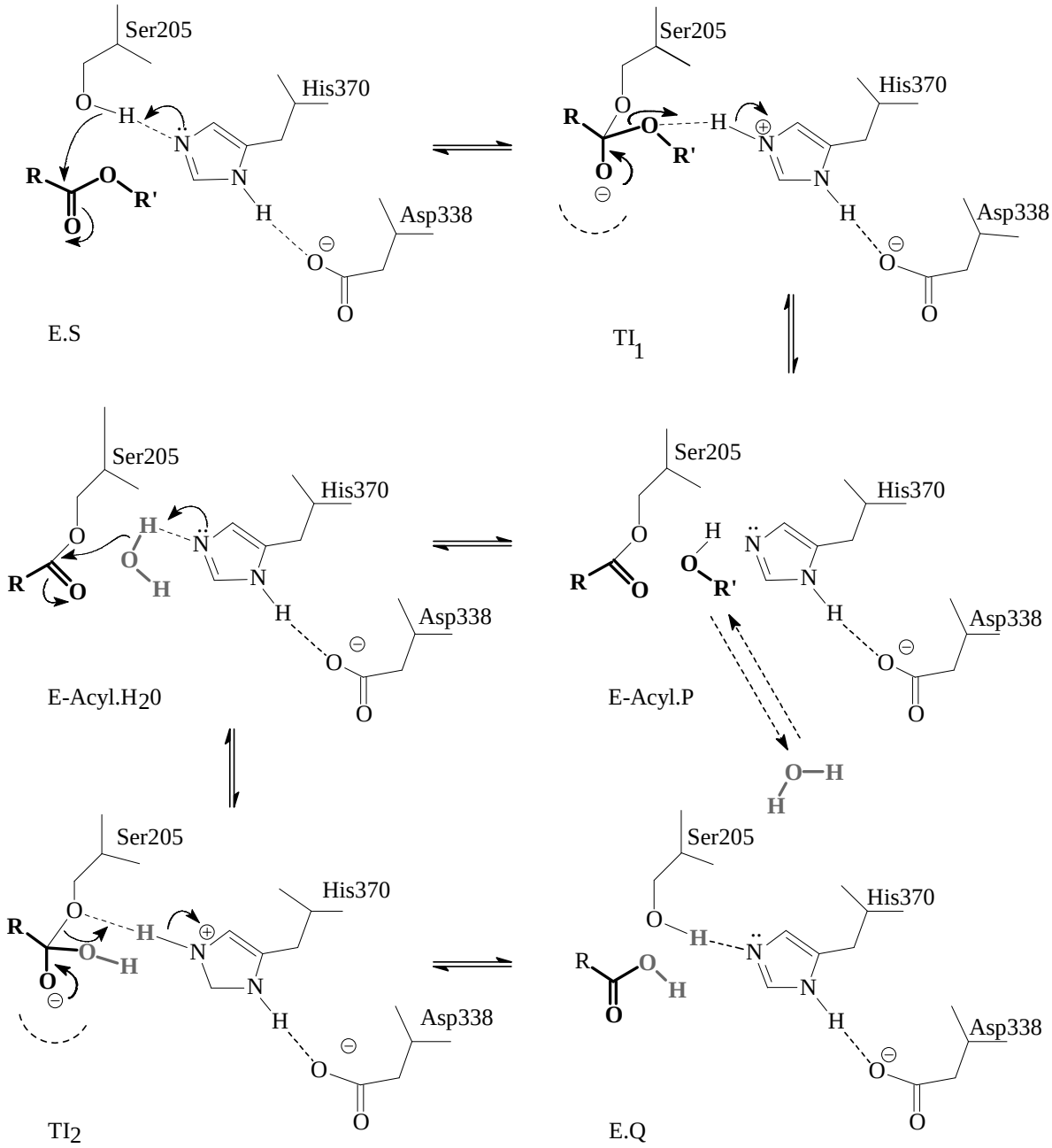

Figure 1. Proposed reaction mechanism for the AEH from *A. turbidans* ATCC 9325. In the first step a covalent acyl-enzyme intermediate is formed. Its deacylation by H_2O results in the net hydrolysis of the substrate. Replacement of water by a nucleophilic β-lactam nucleus can lead to synthesis of a β-lactam antibiotic. In the substrate RCOOR', R and R' are variable groups. TI_1 and TI_2, tetrahedral intermediates 1 and 2. The dotted line represents the oxyanion hole. The figure was freely adapted from Fersht (1985).

A. turbidans form the oxyanion hole via their backbone NH and their side-chain hydroxyl group, respectively (Barends *et al.*, 2003b; Polderman-Tijmes *et al.*, 2002b).

The AEHs can synthesize β-lactam antibiotics from an esterified acyl or side chain donor and the free β-lactam nucleus. In such a kinetically controlled synthesis, the enzyme

catalyzes two other reactions next to the transfer

$$\alpha = \frac{\left(k_{cat} / K_M \right)_Q}{\left(k_{cat} / K_M \right)_{AD}} \qquad [1]$$

of the acyl group to a β-lactam nucleus (aminolysis): the hydrolysis of the side-chain donor to the free acid and the hydrolysis of the formed product. The hydrolysis and aminolysis proceed via the same acyl-enzyme intermediate (Duggleby *et al.*, 1995; Nam *et al.*, 1985; Polderman-Tijmes *et al.*, 2002b; Takahashi *et al.*, 1974). The progress of a kinetically controlled reaction is characterized by the temporary accumulation of a product (Q_{max}). Next to the initial concentrations of the acyl donor and the incoming nucleophilic β-lactam group, two important parameters determine the course of the reaction, i) the rate of the acyl donor conversion vs. product conversion and ii) the rate of acyl transfer to a β-lactam nucleophile vs. that to water (Alkema *et al.*, 2002; Kasche, 1986). The first parameter implicates that the enzyme should have a higher specificity for the acyl donor (AD) than for the antibiotic (Q), thereby reducing the unwanted hydrolysis of the product. This is expressed by the enzyme specificity parameter α (equation 1) (Alkema *et al.*, 2002; Gololobov *et al.*, 1989), which should be small to allow a high level of product accumulation.

The second parameter is determined in the beginning of the reaction when product hydrolysis is negligible and is defined as the ratio of the initial rates of antibiotic synthesis and acyl donor hydrolysis (Vs/Vh). This ratio is dependent on the nucleophile concentration and is determined by the relative reactivity of the nucleophile compared to water (equation 2, (Youshko *et al.*, 2002)).

A high initial V_s/V_h and a high ratio between the concentrations of synthesized antibiotic and hydrolysis product (S/H_{max}) at the moment where product accumulation is maximal (Q_{max}) are indicative for an efficient process with a limited (unproductive) loss of acyl donor.

Although the primary function of Tyr206 was proposed to reside in its main chain NH group, a Tyr206Ala mutant showed a reduced affinity for cephalexin and a lower k_{cat}, resulting in reduced cephalexin hydrolysis by this mutant. This could indicate that mutating the enzyme at this position may yield variants with enhanced potential in biocatalytic cephalexin synthesis (Polderman-Tijmes *et al.*, 2002b). To obtain further insight in the catalytic role of Tyr206 and to explore the possibility to improve the synthetic properties of the AEH, six mutants were made in which Tyr206 was replaced by another amino acid. For each mutant we measured the kinetic properties for the hydrolysis of cephalexin, ampicillin and phenylglycine methyl ester (PGM) to determine the substrate vs. product selectivity of the enzyme (parameter α). Subsequently, the performance of the enzyme in the synthesis of cephalexin and ampicillin was tested and the initial V_s/V_h ratio, the Q_{max}, and the S/H_{max} were determined. The data showed that mutant Tyr206Asn has a higher specificity for the acyl donor than for the product and improved properties in cephalexin and ampicillin synthesis compared to wild type.

$$\frac{V_s}{V_h} = \beta . n \qquad \beta = \frac{\beta_0}{1 + \beta_0 \gamma n} \qquad [2]$$

MATERIALS AND METHODS

Materials

D-2-Nitro-5-[(phenylglycyl)amino]benzoic acid (NIPGB) was obtained from Syncom (Groningen, The Netherlands). D-Phenylglycine methyl ester (PGM), 7-aminodesacetoxy-cephalosporanic acid (7-ADCA), 6-aminopenicillanic acid (6-APA), ampicillin and cephalexin were provided by DSM Life Sciences (Delft, The Netherlands). D-Phenylglycine (PG) was purchased from Acros Organics (Geel, Belgium). The oligonucleotides used for site-directed mutagenesis were provided by Eurosequence BV (Groningen, The Netherlands). All chemicals used in DNA manipulation procedures were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and used as recommended by the manufacturer.

Bacterial strains, plasmids, growth conditions and purification

The host strain *E. coli* TOP10 (Invitrogen, Breda, The Netherlands) was used for propagation of pBADAT (Polderman-Tijmes *et al*., 2002b) and derivatives thereof. For vector isolation the *E. coli* strains were grown at 30 °C on LB medium supplemented with ampicillin. For expression, strains harboring pBADAT and derivatives thereof were grown for 60 h at 14 °C in the presence of ampicillin (Amp, 50 µg/ml) and 0.01 (Tyr206Ala, Tyr206Phe, Tyr206Asn, and Tyr206Trp) or 0.001% (Tyr206Val and Tyr206Lys) arabinose. The wild-type AEH and its mutants were expressed with an N-terminal poly-His tag (His_6) and purified to SDS homogeneity as described before (Polderman-Tijmes *et al.,* 2002b). The His_6-tagged enzyme will be referred to as wild type (WT) unless stated otherwise.

Site-directed mutagenesis and sequencing

Mutants were constructed using the QuickChange site-directed mutagenesis kit of Stratagene (La Jolla, CA, USA) according to the procedure recommended by the manufacturer, starting from the wild-type construct pBADAT (Polderman-Tijmes *et al*., 2002b). When possible, a restriction site was introduced in the mutagenic primers (Table 1). The presence of the mutations and the integrity of the rest of the gene were verified by DNA sequencing at the Department of Medical Biology of the University of Groningen.

Activity assays and determination of kinetic constants

To facilitate an easy quantification of the activity of the enzymes that have a mutation at position 206, their initial hydrolysis rate of the chromogenic substrate NIPGB was determined (Polderman-Tijmes *et al.,* 2002a). One unit is defined as the amount of enzyme needed to convert 1 µmol of NIPGB per min.

For the synthesis of cephalexin and ampicillin, the enzyme was incubated with 15 mM PGM and 30 mM of β-lactam nucleus (7-ADCA or 6-APA) in 50 mM Na-phosphate buffer (pH 6.2) at 30 °C. In these conversions, the amino acid ester functions as the acyl donor and the β-lactam nucleus as the deacylating nucleophile (Fig. 1). The hydrolysis and synthesis of cephalexin and ampicillin and the hydrolysis of PGM were followed by HPLC as described before(Polderman-Tijmes *et al*., 2002a). Enzyme was used at a concentration that catalyzed the hydrolysis of PGM with an initial rate of 0.20 to0.30 mM/min. To obtain the rate of enzymatic PG production, the observed rate of PG

Table 1. Synthetic oligonucleotides used for site-directed mutagenesis of *A. turbidans*[1].

Oligonucleotide sequence 5' → 3'	Mutation
G GGT ATG ACA GG**G** TCG TCC **GC**T GAG GGC TTT ACT G	Tyr206Ala
G GGT ATG ACA GG**G** TCG TCC T**T**T GAG GGC TTT ACT G	Tyr206Phe
G GGT ATG ACA GG**G** TCG TCC **GT**T GAG GGC TTT ACT GTT G	Tyr206Val
GGT ATG ACA GG**G** TCG TCC **G**AT GAG GGC TTT ACT G	Tyr206Asp
GTG GGT ATG ACA GG**G** TCG TCC **A**A**G** GAG GGC TTT ACT GTT G	Tyr206Lys
GGT ATG ACA GG**G** TCG TCC **A**AT GAG GGC TTT ACT GTT G	Tyr206Asn
G GGT ATG ACA GG**G** TCG TCC T**GG** GAG GGC TTT ACT GTT G	Tyr206Trp

[1]) Only the sense primers are shown. The *Asp*I restriction sites are underlined. Mutations are shown in bold.

production was corrected for the first order chemical hydrolysis of PGM. The relative rate of antibiotic formation vs. hydrolysis of PGM (initial V_s/V_h) was determined from the initial slope of antibiotic formation divided by the rate of formation of hydrolysis product (PG). The rates were measured at less than 10% conversion of PGM to minimize the influence of product hydrolysis. The kinetic parameters K_M and k_{cat} were calculated using nonlinear regression fitting (Scientist, Micromath) with Michaelis-Menten and substrate inhibition kinetics. The calculated parameters are given with their standard deviations. The maximal product concentration (Q_{max}) was determined by following the concentrations of cephalexin and PG over time, until the product concentration started to decrease. At Q_{max}, the ratio between the amount of acyl donor converted to antibiotic and the amount hydrolyzed to amino acid (S/H_{max}) was determined. All measurements were at least performed in duplicate, and averages with standard deviation are given.

RESULTS AND DISCUSSION

Production and activity of Tyr206 mutants

The preliminary observation that the alanine mutant of Tyr206 showed a lowered specificity (k_{cat}/K_M) for the antibiotic cephalexin (Polderman-Tijmes *et al*., 2002b), which could be favorable for synthetic reactions, prompted us to further explore the role of this residue. Therefore, Tyr206 was mutated to amino acids with specific properties, i.e. a negative charge (Asp), positive charge (Lys), large and hydrophobic (Trp), polarisable (Asn), small (Val) and similar to tyrosine but then without the hydroxyl group (Phe). To facilitate purification the AEH mutants were expressed with a poly His-tag (His_6) attached to their C-terminus using a pBAD expression system (Polderman-Tijmes *et al*., 2002b). The His_6-tag did not cause a significant change of the K_M or k_{cat} in cephalexin hydrolysis compared to untagged wild-type enzyme and therefore the influence of the tag was considered negligible (Polderman-Tijmes *et al*., 2002b).

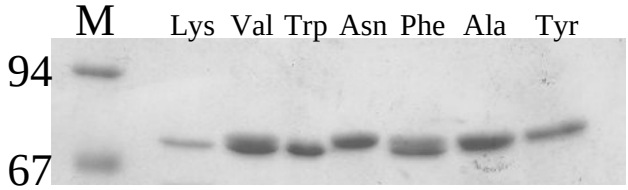

Figure 2. SDS-PAGE of purified AEH-His$_6$ and Tyr206 mutants. Lane M represents part of the molecular weight marker with the masses (kDa) indicated at the left. The lanes correspond to the residues at position 206, thus lane Tyr represents the AEH-His$_6$ wild type.

All the mutant proteins were brought to expression and purified to SDS-PAGE homogeneity (Fig. 2), except for mutant Tyr206Asp, of which the expression level under different induction conditions was too low to purify sufficient protein for the determination of the kinetic parameters. The subunits of Tyr206Ala and Tyr206Trp have the same size as those of WT, indicated by the single band at 72 kDa. The mutants Tyr206Val and Tyr206Phe showed an additional slower migrating protein band with a calculated size of 76 kDa. At the same height as this slower migrating band, the single protein band of mutant Tyr206Asn was observed. The difference in mass of the subunits might be due to a different or lacking N-terminal processing of the 40 amino acids long (4.1 kDa) signal sequence of AEH (Polderman-Tijmes *et al.*, 2002a) Alternatively, mutant Tyr206Asn might be processed correctly but have a slightly reduced mobility due to the mutation. It is known that some point mutations can have an influence on the mobility of a protein on SDS-PAGE (Bak, 1987; Erdjument *et al.*, 1988).

All mutants were still active on NIPGB (Table 2). However, for all the mutants we see a decrease in activity, especially for Tyr206Val and Tyr206Lys.

Kinetic characterization of substrate hydrolysis by the mutant enzymes

To determine whether the mutations at Tyr206 led to changes in relative specificities towards the acyl donor and the antibiotic (factor α), the kinetic parameters for the acyl donor (PGM) and the product cephalexin were determined and compared to that of the wild-type enzyme. All the mutants showed a decrease in k_{cat} and an increase in K_M for both PGM and cephalexin. The decrease in k_{cat}/K_M was largest for cephalexin and especially for the mutants Tyr206Asn and Tyr206Trp this resulted in a favorable decrease of the factor α (Table 3, α = 0.14) hinting at an improved synthesis. For the wild-type tagged enzyme we find a K_M for PGM of 7 mM. This values differs from what was previously reported for the untagged wild-type enzyme (Polderman-Tijmes *et al.*, 2002a), but the earlier values did not take substrate inhibition in account, which at that time was not noticed due to the lower concentrations at which measurements were done

Table 2. NIPGB activities of wild-type AEH and Tyr206 mutants.

Enzyme	NIPGB (μmol/min/mg)
WT	1.16 ± 0.05
Y206A	0.29 ± 0.04
Y206F	0.42 ± 0.05
Y206N	0.068 ± 0.001
Y206W	0.03 ± 0.01
Y206V	0.0005 ± 0.00001
Y206K	0.0019 ± 0.0001

The initial NIPGB activity was measured using 15 mM NIPGB in a 50 mM Na-phosphate buffer (pH 6.2) at 30 °C.

Table 3. Kinetic parameters of cephalexin and PGM hydrolysis for wild-type AEH and the Tyr206 mutants.

	Cephalexin			PGM			α
Enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}.mM^{-1}$)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}.mM^{-1}$)	
WT	274 ± 7	0.45 ± 0.06	609 ± 83	1067 ± 56	1 ± 0.1	1067 ± 121	0.6 ± 0.1
Tyr206Ala	120 ± 2	4.0 ± 0.2	30 ± 2	655 ± 39	5.3 ± 0.9	124 ± 22	0.24 ± 0.05
Tyr206Phe	77 ± 3	2.2 ± 0.3	35 ± 5	2333 ± 198	14 ± 3	167 ± 38	0.21 ± 0.02
Tyr206Asn	37 ± 3	11 ± 2	3.4 ± 0.7	154 ± 7	6.3 ± 0.8	24 ± 3.3	0.14 ± 0.01
Tyr206Trp	14.8 ± 0.9	5.6 ± 0.9	2.6 ± 0.5	388 ± 31	21 ± 4	18 ± 3.8	0.14 ± 0.01
Tyr206Val	12.4 ± 0.1	4.2 ± 0.2	3.0 ± 0.1	1765 ± 94	124 ± 14	14 ± 2	0.21 ± 0.04
Tyr206Lys	1.50 ± 0.05	15 ± 1	0.10 ± 0.01	>15	>100	>0.15	<0.6

Synthesis of antibiotics by mutant enzymes

To evaluate the effect of the improved α-factor on antibiotic production the synthesis of cephalexin was measured for the different mutants. All the Tyr206 mutants had an improved α parameter. This led to a higher initial V_s/V_h ratio and a higher Q_{max} compared to wild type for the Tyr206Ala, Tyr206Asn and Tyr206Lys mutant enzymes (Table 4). In addition to that, the Tyr206Asn mutant showed a better S/H_{max}, resulting in an overall improved cephalexin synthesis compared to wild-type enzyme (Fig. 3). Thus, mutation of Tyr206 led to three mutants with improved synthetic properties. Surprisingly, despite its very low α factor, Tyr206Trp did not perform better then wild-type in cephalexin synthesis. Apparently, the other parameters that influence the initial V_s/V_h and Q_{max}, are not favorable for improved antibiotic synthesis. To understand the effects of the mutations on the

Table 4. Cephalexin synthesis by wild-type AEH and the Tyr206 mutants.

	Cephalexin synthesis[1]			
Enzyme	Cex_{ini} (μmol/min/mg)	Initial V_s/V_h	Q_{max} (mM)	S/H_{max}
WT	1300 ± 500	2.4 ± 0.8	5.4 ± 1	1.8 ± 0.5
Tyr206Ala	300 ± 100	9 ± 2	8.6 ± 0.6	1.6 ± 0.1
Tyr206Phe	70 ± 8	1.9 ± 0.1	7.4 ± 0.1	1.2 ± 0.1
Tyr206Asn	2.2 ± 0.1	3.5 ± 0.3	8.9 ± 0.1	2.8 ± 0.6
Tyr206Trp	100 ± 10	2.1 ± 0.1	5.5 ± 0.1	1.0 ± 0.1
Tyr206Val	140 ± 20	1.0 ± 0.1	5.8 ± 0.4	0.8 ± 0.1
Tyr206Lys	50 ± 10	3.8 ± 0.2	7.9 ± 0.1	1.8 ± 0.1

[1] Cephalexin synthesis from PGM and 7-ADCA (see Materials and Methods).

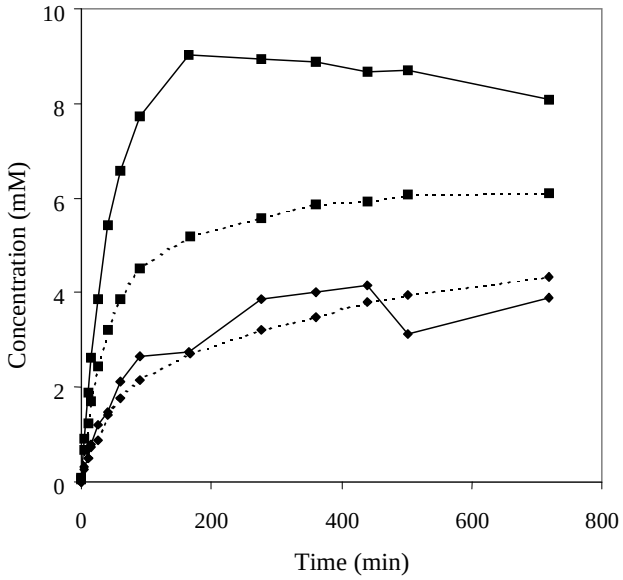

Figure 3. Kinetically controlled synthesis of cephalexin using wild-type AEH (---) and the Tyr206Asn (—) mutant. Cephalexin synthesis from PGM and 7-ADCA (see Materials and Methods). Symbols: (■), cephalexin; and (◆), PG.

synthetic reaction in more detail these parameters must be determined, which requires measurment of the nucleophile-concentration dependence of the initial V_s/V_h.

The initial synthesis activity of cephalexin by WT of 1300 µmol/min/mg measured in this study is in agreement with the value given in the literature (Ryu and Ryu, 1988). The relative decrease seen in the initial synthesis activity (Table 4) for the individual mutants is the same as that observed for the rate of NIPGB hydrolysis (Table 2), except for the mutant Tyr206Val, for which a higher rate of synthesis was found than expected from the NIPGB hydrolysis activity. To investigate the influence of another nucleus (6-APA instead of 7-ADCA) on the catalytic properties of the mutants, we measured the hydrolysis and synthesis of ampicillin using the Tyr206Asn and Tyr296A mutants, which were better in cephalexin synthesis than the wild type. The mutants had a higher K_M and a reduced k_{cat} for ampicillin compared to wild type. Thus, also in this case the specificity for the antibiotic was lowered more than that for PGM, which resulted in more beneficial α values, especially for Tyr206Asn AEH that has a very low affinity for ampicillin (Table 5). From a comparison between the changes in K_M and k_{cat} for ampicillin with those measured for cephalexin, we see that the effect of the mutations on the conversion of these substrates are different. The mutations have a much larger effect on the k_{cat} of ampicillin than on the k_{cat} for cephalexin, while for the K_M it is the other way around. For the mutants a slight improvement of V_s/V_h and a small but significant increase in Q_{max} and S/H_{max} compared to the wild-type enzyme were measured for ampicillin synthesis (Table 5). Apparently, at the same nucleophile

Table 5. Ampicillin hydrolysis and synthesis for AEH (WT), Tyr206Ala and Tyr206Asn.

	Ampicillin hydrolysis				Ampicillin synthesis[1]		
Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)	α	Initial V_s/V_h	Q_{max}	S/H_{max}
WT	0.6 ± 0.1	339 ± 17	565 ± 98	0.53	1.8 ± 0.1	5.7 ± 0.1	1.03 ± 0.04
Tyr206Ala	1.8 ± 0.4	59 ± 4	33 ± 8	0.27	1.7 ± 0.1	6.9 ± 0.1	1.12 ± 0.02
Tyr206Asn	4.5 ± 0.5	8 ± 0.3	1.8 ± 0.2	0.08	2.1 ± 0.3	7.35 ± 0.07	1.14 ± 0.01

[1] Ampicillin synthesis from PGM and 6-APA (see Materials and Methods).

concentrations the relative reactivity (β, eq. 2) of the 6-APA nucleus is lower than of the 7-ADCA nucleus, which is evident from the lower V_s/V_h ratios found during the synthesis of ampicillin. The increase in V_s/V_h for the Tyr206Asn mutant might point to an improved interaction with the incoming nucleus for coupling.

In conclusion, the Tyr206Asn mutant AEH showed improved performance with respect to the synthesis of ampicillin and cephalexin.

Structural analysis

The recently solved crystal structure of the wild-type *A. turbidans* AEH, Tyr206Ala and Ser205Ala mutants by Barends *et al.* (2004) provides insight into the structure-function relationship of the AEHs. In the structures the phenyl ring of Tyr206 points away from the active site and is situated in a hydrophobic pocket formed by residues Pro111, Trp209 and Met231. This can explain why charged residues like aspartate and lysine are not readily accepted at the Tyr206 position in *A. turbidans* and result in almost inactive mutants. The authors describe that in the Tyr206Ala mutant the backbone conformation (important for the oxyanion stabilization by the backbone NH of Tyr206) did not change as compared to wild-type enzyme. Via different interactions, however, the mutation caused a small shift (1 Å) of the side chain from second oxyanion hole contributor (Tyr112) towards the Oγ of the catalytic serine corresponding to a weak hydrogen bond. The resulting close interaction between the tyrosine and serine is expected to lower the nucleophilicity of the serine explaining the reduced k_{cat} values for the Tyr206 mutants.

At the same time Tyr112 has hydrophobic interactions with the β-lactam moiety of an antibiotic as revealed by the crystal structure of the Ser205Ala mutant of *A. turbidans* AEH co-crystallized with ampicillin (Barends *et al.*, 2004). This explains the different effects of the Tyr206 mutations towards ampicillin and cephalexin, which only differ in their β-lactam nucleus (Tables 3 and 5). However, the positive effect observed upon the introduction of the Asn side chain on position 206 can not directly be explained from the currently available structures.

In conclusion, we have shown that the synthetic properties of the AEH from *A. turbidans* can be enhanced by site-directed mutagenesis as the Tyr206Asn mutant AEH showed much less hydrolysis and an improved level of transient accumulation in the synthesis of both ampicillin and cephalexin. A possible structural explanation for the observed properties of the mutants remains speculative in the absence of an X-ray structure with ampicillin bound in a productive mode. However, the observed effects could be related to the fact that residue Tyr206 takes part in the formation of the oxyanion hole and indirectly influences interactions of the enzyme both with the leaving group moiety of the acyl donor and with the incoming nucleophile. Additionally, further insight in the nucleophilic reactivity (factor β) is needed to explain the observed kinetic effects in more detail. In the mean time, a directed evolution approach starting from Tyr206Ala and/or Tyr206Asn may be used to obtain further improved enzymes.

7

Summary and conclusions

The α-amino acid ester hydrolases and their future

INTRODUCTION

This thesis describes the genetic and biochemical characterization of the α-amino acid ester hydrolases (AEHs), enzymes applicable in the biocatalytic production of semi-synthetic β-lactam antibiotics. These enzymes are homotetramers with subunits of 70-72 kDa and can catalyze the synthesis and hydrolysis of α-amino substituted β-lactam antibiotics. At the start of this work, little was known about the AEHs. A much better studied enzyme that is able to perform the antibiotic coupling reaction is the penicillin acylase from *Escherichia coli* (PA). This heterodimeric periplasmic protein consists of a small α subunit of 23 kDa and a β subunit of 63 kDa. It is produced from an inactive precursor through autoproteolytic processing by a serine residue that becomes the N-terminus of the β-subunit. The serine oxygen acts as a nucleophile and its N-terminal amine group as a proton donor in the catalytic mechanism of PA (Duggleby *et al*., 1995). Therefore, PA was identified as a so-called N-terminal nucleophile (Ntn) hydrolase. These enzymes have a serine, threonine or cysteine as N-terminal nucleophile and share a common fold. Although they do not share significant amino acid sequence identity among each other or with PA, other β-lactam acylases, such as penicillin V acylase from *Bacillus sphaericus* and cephalosporin acylase from *Pseudomonas diminuta*, also belong to this family.

The β-lactam antibiotic synthesis reactions catalyzed by PAs and AEHs are dependent on an activated precursor of the acyl group, usually an ester or amide, of which the acyl group is transferred via an acyl-enzyme intermediate to a free β-lactam nucleus. These conversions are kinetically controlled, since they are dependent on the transient accumulation of a coupling product that does not represent the thermodynamically most stable situation.

The AEHs have interesting biocatalytic properties for the synthesis of α-amino substituted semi-synthetic β-lactam antibiotics. For example, due to their preference for esters, it is expected that the relative affinity for the product (amide) compared to the acyl donor (ester) is lower than for PA, which is primarily an amidase. Conceivably, a higher level of product accumulation can be reached in a kinetically controlled synthesis reaction with AEHs than with PA. Furthermore, the lower pH optimum of the AEHs (pH 6 compared to pH 7.5-8 for PA) is advantageous for the stability of β-lactam nuclei.

Prior to our study, not much was known about the biochemical properties of the AEHs. The reported subunit composition and subunit sizes were inconclusive, the cellular localization was unclear, the catalytic mechanism unknown and the genes had not been cloned. In this chapter an overview of the results leading to the disclosure and characterization of a new class of β-lactam acylases, the AEHs, will be presented. Additionally, possible strategies to obtain further insight in the structure-function relationship of and/or to improve the AEHs will be given.

THE α-AMINO ACD ESTER HYDROLASES

Cloning of the AEHs

Based the results from a comparison of AEHs and other β-lactam acylases in the synthesis of ampicillin from an activated acyl side chain and a free β-lactam nucleus (kinetically controlled synthesis) (*Chapter 2*), we selected the AEHs

from *X. citri* IFO 3835, *A. turbidans* ATCC 9325 and *A. pasteurianus* ATCC 6033 for further studies. To enable detailed characterization of these enzymes, larger amounts of protein were needed and therefore our first goal was to clone their genes. Activity screening of genomic cosmid libraries of the selected strains with various methods was unsuccessful (*Chapter 2*). Finally, amino acid sequencing of the purified AEHs allowed cloning via Southern hybridization techniques of the corresponding *aeh* genes from *A. turbidans* and *X. citri*, as described in *Chapter 3* and *4*, respectively. Due to the low expression level of the AEH in *A. pasteurianus* we were unable to purify this enzyme from the original host. However, by screening the genomic library of *A. pasteurianus* with a DNA probe based on the sequence of the AEH from *A. turbidans*, the *aeh* gene from *A. pasteurianus* was cloned as well (unpublished result).

The *aeh* genes were subsequently placed in several *E. coli* expression vectors and in all cases active protein was produced. Therefore, the reason why the genes could not be detected in the cosmid libraries upon screening for hydrolysis or synthesis activity (*Chapter 2, 3 and 5*) was probably lack of expression. Analysis of the sequences upstream of the *aeh* genes revealed a possible ribosome binding site (although located unfavorably) but promoter sequences that are known to be recognized in *E. coli* could not be identified (Fig. 1). The highest level of AEH protein was achieved with a pBAD vector (arabinose promoter), in which expression of the *A. turbidans* AEH was 5-fold higher than in the wild-type organism, with 1% of the total soluble protein of *E. coli* being AEH (compared to 0.2% in the original organism). The cloning and expression of the *aeh* genes in *E. coli* facilitated the purification of sufficient amounts of enzyme for kinetic and structural analysis.

Sequence analysis

The amino acid sequences of the AEHs from *X. citri* and *A. turbidans* are identical for 60%. The sequences from *A. pasteurianus* and *A. turbidans* have an identical length and only differ at position 83 and 641 (an Ala and a Val in *A. pasteurianus* and a Pro and an Ala in *A. turbidans*). The high degree of similarity between the latter two is in agreement with their comparable activity with respect to ampicillin hydrolysis (*Chapter 2*). A similarity search with the AEH amino acid sequences of the protein databases revealed many homologous proteins. However, no homology with other known β-lactam acylases was found, except with the glutaryl 7-aminocephalosporanic acid acylase

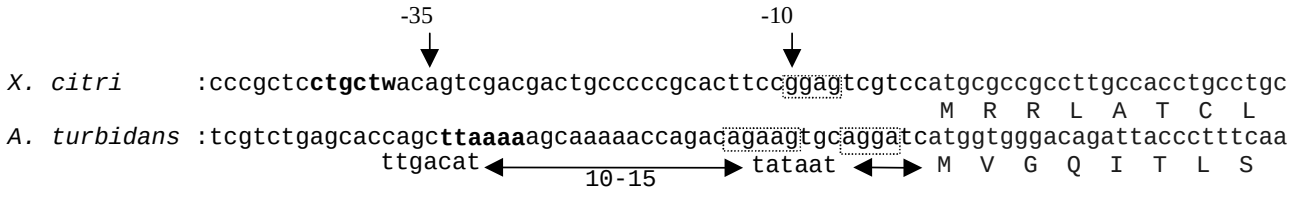

Figure 1. **Sequences upstream of the *aeh* genes.** Shown are parts of the cosmid inserts harboring the *aeh* genes of *X. citri* and *A. turbidans*. Promoter sequences ideally should be located at –35 and –10 with respect to the initiation codon (atg). Consensus sequences for the individual positions of *E. coli* promoters are given. Possible –35 promoter sequences are indicated in bold. Ribosome binding sites are located ideally around –10 with respect to the initiation codon and should be a five to eight bases of purine-rich sequence. Possible ribosome binding sites are boxed.

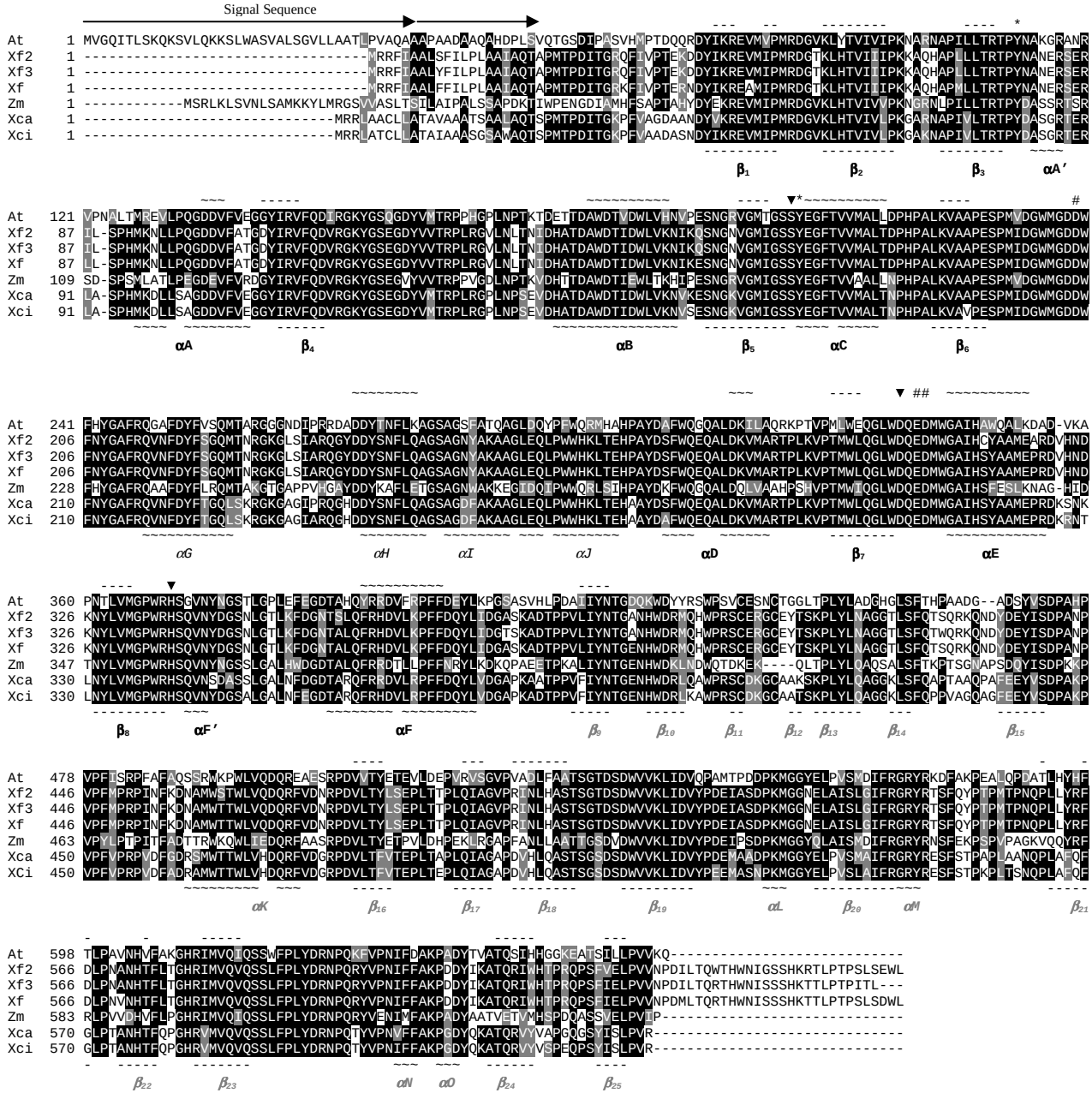

Figure 2. Sequence alignment of the cloned and some putative α-amino acid ester hydrolases. The alignment was made using Clustal W 1.8. The abbreviations At and Xci refer to the AEHs from *A. turbidans* ATCC 9325 and *X. citri* IFO 3835, respectively; Xf, to *X. fastidiosa* (accession no. AAF83839); Xf2, *X. fastidiosa* Ann, GI no. 22996819; Xf3, *X. fastidiosa* Dixon, GI no. 22993959; Xca, *X. campestris* pv. *campestris* (accession no. AAM41516); and Zm, *Z. mobilis* (accession no. AAD29644). The arrows indicate the predicted signal sequences. The first arrowhead indicates the cleavage site for the *A. turbidans* AEH and the second for all the other enzymes. ~~~ stands for α-helices, -----, for β-strands. The nomenclature of the structural elements was adopted from Barends *et al.* (2003b). The predicted structural elements are indicated above the sequence of *A. turbidans.* Below the *X. citri* sequence the structural elements derived from the structure (PDB no. 1MPX) are shown. Symbols: ▼, catalytic triad residues; *, oxyanion-hole residues, +, Trp residue interacting with the carboxylate cluster and #, α-amino pocket residues. The structural elements of the α/β-hydrolase fold are depicted in bold, those of the cap domain in italics and those of the jellyroll domain in grey.

from *Bacillus laterosporus* (26% identity). This enzyme consist of a single polypeptide chain (70 kDa) but does not show the high specificity towards α-amino compounds, nor has it been described that it can synthesize β-lactam antibiotics (Aramori *et al*., 1991b). Apart from the cocaine esterase from a *Rhodococcus* sp. (Larsen *et al*., 2001), all the other homologous proteins originated from genome sequencing projects and their enzymatic properties are still unknown (*Chapter 3*). Seven proteins present in the protein databases have 60% or more sequence identity with the AEHs: two proteins from *Xanthomonas* sp., four from *Xylella fastidiosa*, and one from *Zymononas mobilis*. All these proteins are annotated in the database as putative glutaryl 7-aminocephalosporanic acid acylases based on their identity (on average 25%) with the enzyme from *B. laterosporus*. However, experiments with the putative glutaryl acylase from *Z. mobilis* (*Chapter 5*) showed that this enzyme is more likely an AEH as it can synthesize and hydrolyze cephalexin and needs the α-amino group on the substrate for activity. All putative AEHs (Fig. 2), except for the one from *Z. mobilis*, are predicted to have an N-terminal signal sequence and therefore can be designated as periplasmic proteins (Nakai, 2000; Nielsen *et al*., 1997). This conclusion is based on the presence of typical features for signal peptides, such as positively charged residues at the N-terminus followed by a stretch of hydrophobic residues. There is also a consensus pattern for cleavage by a periplasmic signal peptidase (AXA). The putative signal sequence varies in length from 18 residues in the *Xylella* strains to 40 residues in *A. turbidans*. The prediction of the N-terminal sequence for *A. turbidans* was in agreement with the determined N-terminal sequence of the purified protein. However, localization and subcellular fractionation experiments (data not shown) indicated that the AEH activity from *A. turbidans* was mainly present in the cytoplasmic fraction, which is probably caused by adhesion of the AEH to the cell envelope after translocation (*Chapter 3*). Based on the features of the putative signal sequence the protein of *Z. mobilis* is predicted to be a membrane-associated cyto-plasmic protein, which explains why the AEH activity of *Z. mobilis* was associated with the membrane fragments (*Chapter 5*). The N-terminal sequence is important for the production of active enzyme, since cloning of the *aeh* gene of *A. turbidans* and *Z. mobilis* without it resulted in inactive clones (*Chapter 3*).

Summarizing, we conclude that when a protein is identical to one of the AEHs by 60% or more and shows conservation of the catalytic triad and the carboxylate cluster (see below), it is very likely able to hydrolyze and synthesize α-amino substituted β-lactam antibiotics and thus belongs to the AEH class of the β-lactam acylases family. PCR experiments with *Xanthomonas rubrilineans* (Krest'ianova *et al*., 1990) and *Achromobacter* sp. (Fujii *et al*., 1976) (*Chapter 5*) showed that the esterases expressed by these organisms also belong to the family of AEHs describe here. Taking this in account, it is expected that the AEH class encompasses at this moment at least 10 members based on known substrate specificities, subunit composition or sequence similarities (Fig. 3). The growing amount of data from genome sequencing projects will very likely reveal more AEHs.

Kinetic characterization of AEHs

The substrate range of the AEHs from *X. citri*, *A. turbidans* and *Z. mobilis* was explored and the kinetic parameters for several substrates were

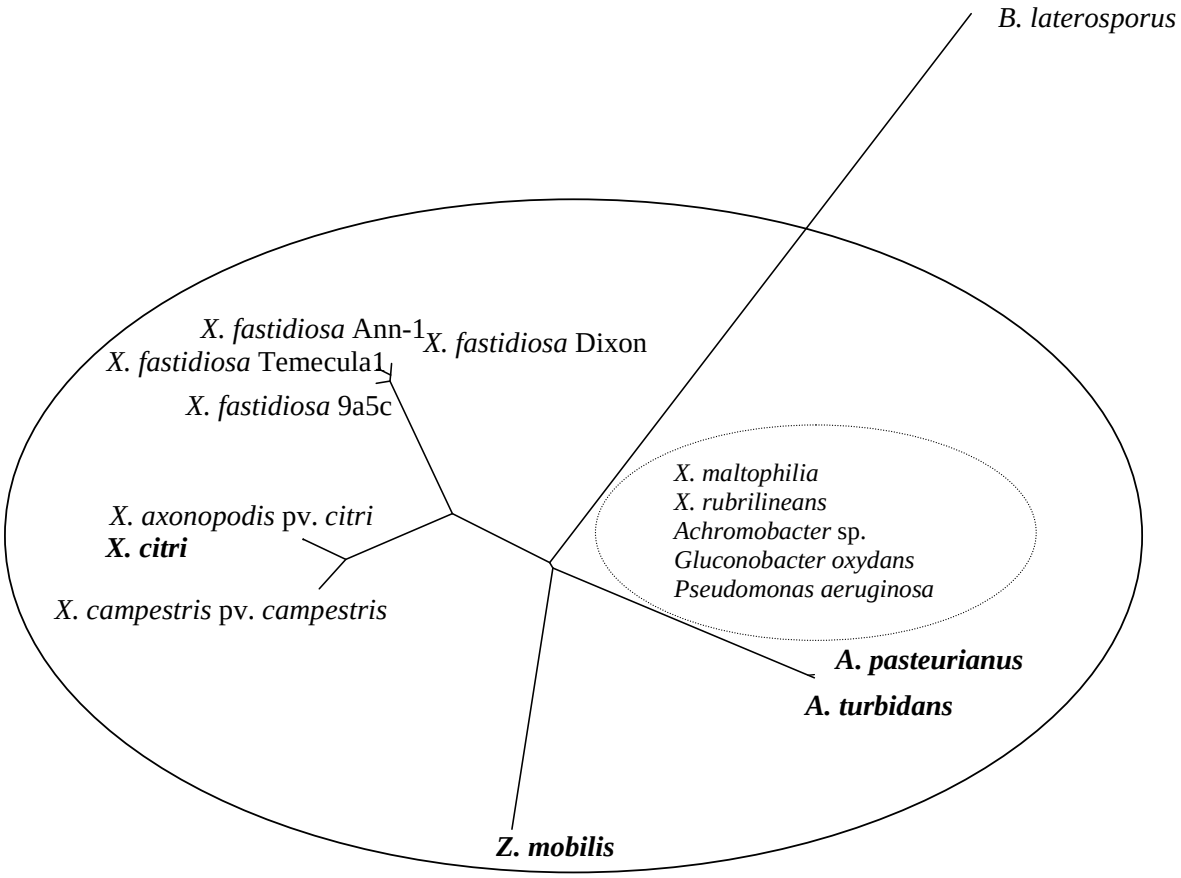

Figure 3. A phylogenetic tree of the AEH sequences and the glutaryl acylase from *B. Laterosporus*: a new class of β-lactam acylases. In the closed circle the sequences with 60% or more identity are given. In bold the AEHs cloned and studied in this study have been indicated. Three sequences are from *Xanthomonas* sp.: *X. axonopodis* pv *citri* 306 (accession no. AAM37193, (da Silva *et al*., 2002); *X. campestris* pv. *campestris* ATCC 33913 (accession no. AAM41516) (da Silva *et al*., 2002); and *X. citri* IFO 3835 (Barends *et al*., 2003b). Four proteins are from *Xylella fastidiosa*: *X. fastidiosa* 9a5c, accession no. AAF83839 (Simpson *et al*., 2000), *X. fastidiosa* Temecula1, accession no AAO28199 (Van Sluys *et al*., 2003); *X. fastidiosa* Ann-1, GI no. 22996819 and *X. fastidiosa* Dixon, GI no. 22993959). One is from *Zymononas mobilis* ZM4 (accession no. AAD29644), *A. turbidans* ATCC 9325 (Polderman-Tijmes *et al*., 2002a) and *A. pasteurianus* ATCC 6033 (this thesis). Other organisms that have enzymes that based on activity profiles are expected to be an AEH, but of which no sequence information is available, are indicated in the dashed circle. For *Gluconobacter oxydans* (Liu and Lee, 1995) and *Pseudomonas aeruginosa* (Wang *et al*., 1990) cephalexin synthesis has been reported. See the text for information about the AEHs from the other strains. The glutaryl acylase from *B. laterosporus* J1 (Aramori *et al*., 1991b) is related to the AEHs (26% sequence identity), but the AEH-typical α-amino carboxylate pocket is not conserved and is therefore not considered an AEH. It is expected that all the enzymes depicted in this tree are α/β-hydrolase fold enzymes.

determined (Table 1). The AEHs from *X. citri* and *A. turbidans* have high turnover numbers (k_{cat}) for D-phenylglycine methyl ester (for comparison: the highest k_{cat} value of PA for a non-chromogenic substrate is obtained with phenylacetic acid methyl ester, 190 s^{-1}). The most striking difference between the AEHs from *X. citri*, *Z. mobilis* and *A. turbidans* is that the first two show a much lower reactivity with *para*-hydroxy-substituted substrates than the latter. Based on sequence alignments and structural analysis a possible explanation is given further on.

Besides the ability to synthesize and hydrolyze β-lactam antibiotics, the AEHs can catalyze the transfer of acyl groups of a variety of D- and L-amino acid methyl esters (Kato *et al.*, 1980; Takahashi *et al.*, 1974) to water and 7-ADCA (*Chapter 2 and 5*). The substrate range for the acceptor of the acyl group has not been studied intensively, but from incubations with D-phenylglycine methyl ester (D-PGM) we do known that the acceptor site is specific for the L-configuration, since the coupling product D-PG- D-PGM was not formed (data not shown). Therefore, the AEHs may be used for the synthesis of dipeptides consisting of a D-amino acid and an L-amino acid. The dipeptides can be interesting for the production of biologically important peptides containing a D-amino acid, such as peptide antibiotics. Further exploration of the substrate specificity of the AEHs is needed to determine the feasibility of this application. The ability to produce D-amino acid containing peptides has been shown for a so-called α-amino acid ester hydrolase from *Bacillus mycoides* (Sugihara *et al.*, 2001). However, this enzyme is a homotetramer of subunits of 39 kDa and no hydrolysis of β-lactam antibiotics has been observed, indicating that it is not an AEH.

Catalytic triad

Extended sequence similarity searches showed that a Gly-X-Ser-Tyr-X-Gly (X = any amino acid) motif is conserved in the AEHs. This motif is also present in X-dipeptidyl amino-peptidases in which it contains the active site serine and is located in an α/β hydrolase fold (Chich *et al.*, 1992) (*Chapter 3*). Surprisingly, the AEHS are not inhibited by the well-known PA and serine hydrolase inhibitor *p*-phenylmethylsulfonyl fluoride (*Chapter 4* and (Nam *et al.*, 1985). However, *p*-nitroguanidinobenzoate (*p*-NPGB) (another serine inhibitor but with an amino group) proved to be a very useful inhibitor. During the reaction of the AEH from *A. turbidans* with *p*-NPGB the acyl-enzyme intermediate accumulates, which is followed by the very slow hydrolysis of the enzyme-inhibitor complex (*Chapter 4*). This confirms the acyl-enzyme catalytic mechanism as proposed in the literature (Blinkovsky and Markaryan, 1993; Nam *et al.*, 1985). Isolation and subsequent mass spectrometry analysis of CNBr fragments of the enzyme-inhibitor complex and the uncomplexed enzyme led to the identification of the active site serine of the AEH from *A. turbidans*. As expected, this residue (Ser205) is located in the Gly-X-Ser-Tyr-X-Gly motif mentioned above (*Chapter 3*). Extended sequence comparisons showed that Ser205 aligns with the serines that are part of the catalytic triad present in different α/β-hydrolase fold enzymes. The other members of the triads (an acid and a base) align with Asp338 and His370 of the AEH from *A. turbidans*. A mutation of these residues to catalytically inert alanines resulted in mutants that have lost the capacity to hydrolyze or synthesize β-lactam antibiotics. This suggested that the N-terminal part of the AEH of *A. turbidans* has an α/β-hydrolase fold structure with a classical catalytic triad of a Ser205, Asp338 and His370. This conclusion was supported by the organization of the predicted structural elements (Fig. 2 and *Chapter 4*) and was confirmed by the crystal structures of the AEHs of *X. citri* and *A. turbidans* (Barends *et al.*, 2003b; Barends *et al.*, 2004).

The general consensus sequence motif used to identify the nucleophile located in the nucleophile elbow in α/β-hydrolase fold enzymes

Table 1. Kinetic parameters of AEH.

***X. citri* AEH**			
Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)
D-2-Nitro-5-[(phenylglycyl)amino]benzoic acid	0.07	0.4	5.7
Cephalexin	1.8	160	89
Ampicillin	1.2	58	48
D-phenylglycine methylester	90	1860	21
D-phenylglycine amide	n.d.	1.6	
Cephadroxil	-	< 0.01	
Amoxicillin	-	-	2[a]
D-4-hydroxyphenylglycine methylester	-	-	9[a]
***A. turbidans* AEH**			
Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.mM^{-1}$)
D-2-Nitro-5-[(phenylglycyl)amino]benzoic acid	1.1	0.4	0.36
Cephalexin[b]	0.45	247	609
Ampicillin[c]	1.0	162	162
D-phenylglycine methylester[b]	1	1067	1067
D-phenylglycine amide[d]	>13	>43	3.3
Cefadroxil[b]	1.7	9.6	5.6
Amoxicillin[b,c]	2.6	10	3.9
D-4-hydroxyphenylglycine methylester	11	263	24
***Z. mobilis* AEH**			
Substrate[d]	K_M (mM)	k_{cat} (s^{-1})	
Cephalexin	2.0	n.d.	
D-phenylglycine methylester	5.6	n.d.	
Cefadroxil	-	< 0.01	
D-4-hydroxyphenylglycine methylester	-	< 0.1	

n.d. Not determined.
\+ Active, but parameters not determined.
\- No measurable activity.
a Determined from initial slope of Michaelis Menten curve.
b Measured with C-terminal His_6-tagged AEH
c Substrate inhibition was observed with K_i of 200 mM (NIPGB) and K_i of 3 mM [d] k_{cat}/K_m was calculated from the initial slope of the Michaelis Menten curve, the error is 30%.
d D-2-Nitro-5-[(phenylglycyl)amino]benzoic acid is converted by *Z. mobilis* but no kinetic parameters were determined.

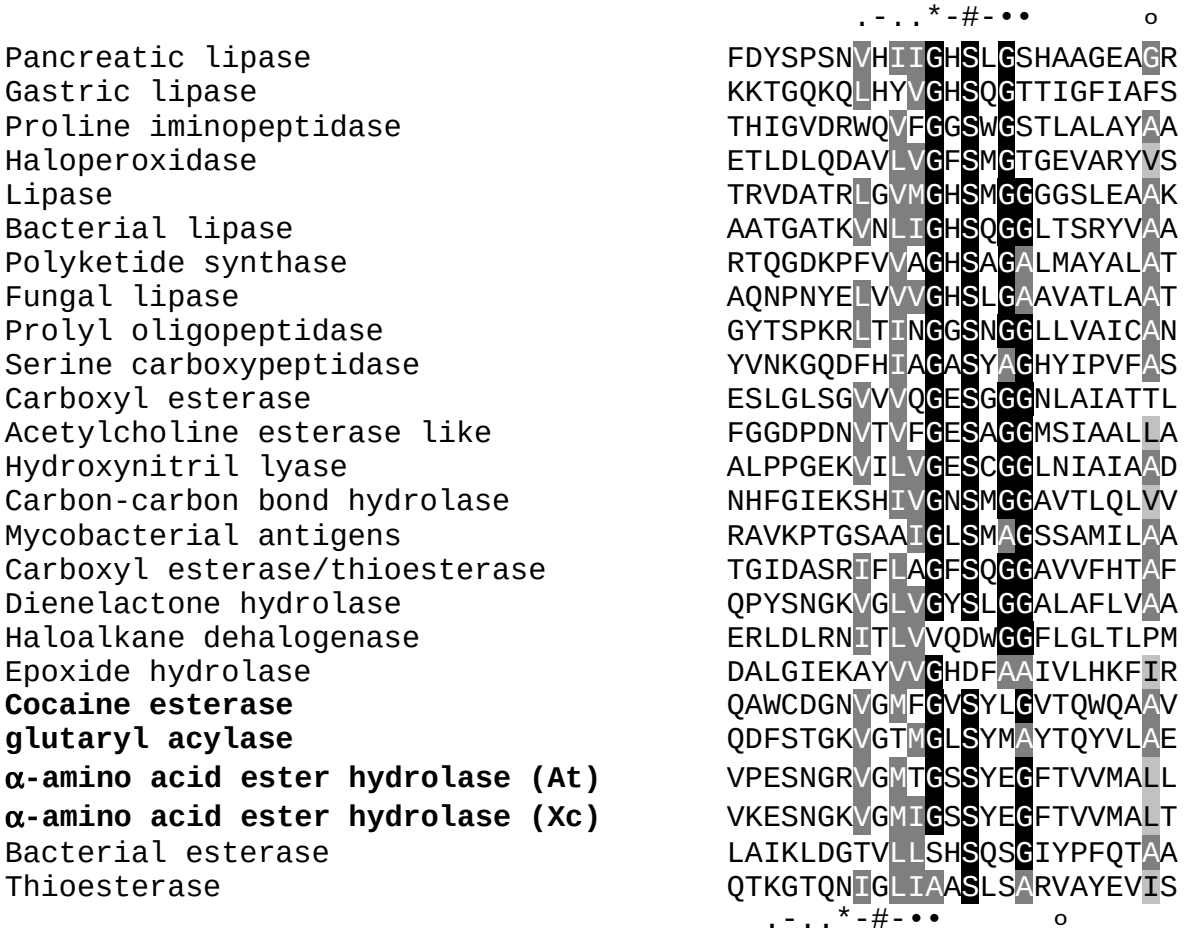

Figure 4. Alignment of the sequences surrounding the catalytic nucleophile of members of all the different α/β-hydrolase fold families including the AEHs and glutaryl acylase. Symbols: (.), possible hydrophobic residue (H:Val, Leu, Ile or Met); (*), small residue (Sm: Gly, Ser, Ala or Val); (#), nucleophile (Nu: Ser, Cys, Asp); (•), possible small residue; (○), small and/or hydrophobic residue (Ala, Val, Leu, Ile); (-), any residue. The sequences used are from proteins that are a member of the indicated families.

is Sm-X-Nu-X-Sm (Sm stands for small residue (Gly or Ala), Nu for nucleophile, and X can be any amino acid). Although the nucleophile elbow in the AEH from *X. citri* has the same structural characteristics as in other α/β-hydrolase fold enzymes (Barends *et al.*, 2003b), the AEHs show a deviation from the consensus sequence surrounding the nucleophile (Fig. 2) as in the AEHs a large glutamate instead of a small residue is located at Nu+2 position. A similar deviation is seen in the myristoyl-ACP-specific thioesterase from *Vibrio harveyi* (Lawson *et al.*, 1994) in which the residue at the Nu+2 position is a serine which is slightly larger than the usual Gly or Ala. To be able to identify the nucleophiles in the thioesterases, a more general consensus motif around the active site nucleophile was defined (Li *et al.*, 1996), Sm-X-Nu-Hl-Sm-Sm (Hl stands for a large hydrophobic residue). However, also this motif does not allow the identification of the catalytic nucleophile in the AEHs from their amino acid sequences. To be able to identify the nucleophile in all α/β-hydrolase fold enzymes, a broader nucleophile consensus sequence is

needed. Therefore, we aligned the sequences surrounding the nucleophiles of members of each α/β-hydrolase fold family (SCOP database) and of the AEHs from *X. citri* and *A. turbidans* and the glutaryl acylase of *B. laterosporus* (Fig. 4). The first small residue (Gly, Ala, Val, Ser) and the nucleophile (Ser, Asp) in Sm-X-Nu-X-Sm are conserved in all the enzymes. Then, after the nucleophile the α/β-hydrolase fold enzymes have a small residue at either position Nu+2, at both Nu+2 and Nu+3 or only at Nu+3 (Fig. 4). Based on this alignment we propose the following consensus sequence which can help to identify α/β-hydrolase fold enzymes and their nucleophile, Sm-X-Nu-X-{Sm}-{Sm}, in which the brackets stand for optional on the condition that one of the two should be present. The extended consensus sequence including the hydrophobic residues (Nu-3, Nu-4 and/or Nu-6 and a small or hydrophobic residue at position Nu+10) located in the core of the strand preceding or the helix following the nucleophile can be useful for confirmation.

Catalytic mechanism

The X-ray structures of the AEH from *X. citri* and *A. turbidans* (Barends *et al.*, 2003b; Barends *et al.*, 2004) confirmed the presence of the catalytic triad and the α/β-hydrolase fold (Fig. 4 and *Chapter 5*). Additionally, ampicillin modeling and binding experiments explained the absolute need of the AEHs for α-amino-substituted substrates. A carboxylate cluster formed by Asp208, Glu309 and Asp310 (Fig. 4, *X. citri* numbering) surrounds the α-amino group of ampicillin. Since at the optimum pH-value of the AEHs (pH 6.2) the α-amino groups of, for example, phenylglycine methyl ester, ampicillin and cephalexin (pK_a's around 7) are mainly present in their charged forms (*Chapter 4*), this negatively charged pocket is apparently needed for substrate binding by electrostatic interactions.

In α/β-hydrolase fold enzymes, the oxyanion hole needed to stabilize the negatively charged tetrahedral intermediate formed during covalent catalysis is usually created by two main chain NH groups. One is donated by the residue that directly follows the nucleophile and the other comes from an amino acid between strand β3 and helix αA (Ollis *et al.*, 1992). Sequence alignments showed that two tyrosines are present at these positions in the AEHs (Fig. 2), which was confirmed by the *X. citri* AEH structure. The tyrosine next to the catalytic nucleophile (TyrA) contributes to the oxyanion hole with its main chain NH as expected. However, the second tyrosine (TyrB) contributes its side chain OH. The oxyanion hole in the related prolyl oligopeptidase (Fülöp *et al.*, 1998) and cocaine esterase (Larsen *et al.*, 2001) is also formed in this way.

Based on the results from the sequence analysis, inhibitor studies, effects of mutations, and the structure, a catalytic mechanism is proposed for the AEHs that involves an acyl-enzyme intermediate as in related α/β-hydrolase fold enzymes (Fig. 5). Once the substrate is complexed to the enzyme, the catalytic triad histidine (base) increases the reactivity of catalytic serine (nucleophile) by polarising the O-H bond through hydrogen bonding. In a concerted process, the activated serine performs a nucleophilic attack on the carbonyl of the substrate, and the tetrahedral intermediate is formed. This intermediate bears a negative charge on the substrate oxygen, which is stabilised by the oxyanion hole. The catalytic triad aspartate (acid) stabilizes the positive charge that develops on the

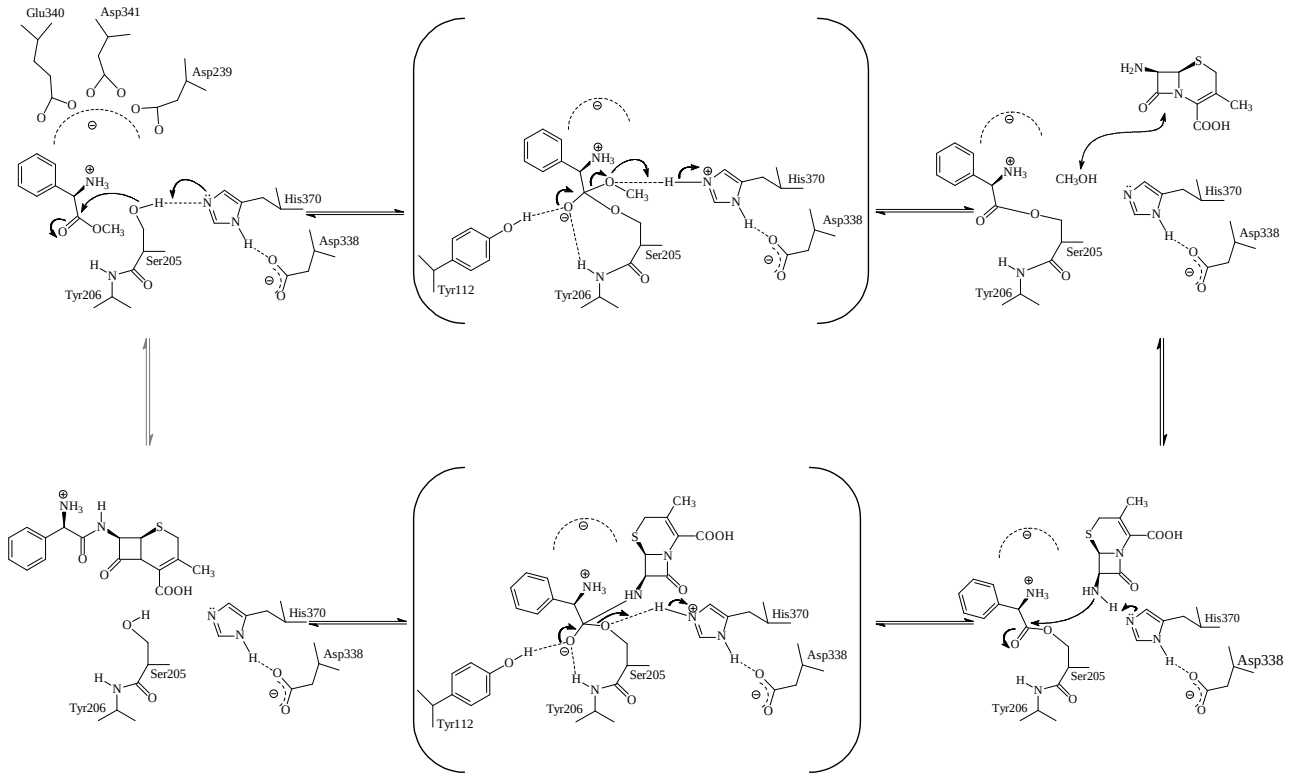

Figure 5. Proposed catalytic mechanism for the α-amino acid ester hydrolases. The figure shows the synthesis of cephalexin from an activated side chain (phenylglycine methyl ester) and the free β-lactam nucleus (7-amino-desacetoxy cephalosporanic acid). The tetrahedral intermediates are indicated between brackets. The amino acids involved are the same for each AEH, the numbers of the AEH from *A. turbidans* are given.

catalytic histidine during formation of the covalent intermediate. The unstable tetrahedral intermediate collapses, leaving the enzyme acylated and releasing the leaving group from the active site, which is probably simultaneously protonated by the histidine. During deacylation of the enzyme a nucleophilic substrate (acyl acceptor) is probably activated by the catalytic triad histidine and performs a nucleophilic attack on the carbonyl carbon atom of the covalent intermediate. The acceptor can either be water (hydrolysis) or a free β-lactam nucleus (aminolysis). In the crystal structure of *X. citri* AEH a water molecule is observed near the catalytic histidine. It is postulated that this water is first bound to the protein at this position where it is activated by the catalytic histidine in order to attack the acyl-enzyme. The β-lactam nucleus (the nitrogen of its amide group) is also activated at this position but the other part of the molecule very likely interacts with the side chain of TyrB at the same time (Barends *et al.*, 2004). After the attack of the acyl-enzyme by either water or the β-lactam nucleus a tetrahedral intermediate is formed which then falls apart, releasing the product and returning the enzyme back to its original state, ready for another round of catalysis. Hydrolysis yields the free acid whereas aminolysis by a β-lactam nucleus leads to a semi-synthetic antibiotic. The transfer of the hydrogen atoms back and forth (Fig. 5) is based on a general mechanism for acyl transfer reactions but should be investigated in more detail, for example by proton inventories for the AEHs in particular. In

the crystal structure of cocaine esterase complexed with an inhibitor, a covalently bound tetrahedral-intermediate analogue has been observed. In this intermediate the catalytic triad residues are in a productive configuration. Upon deacylation of the enzyme, the serine and histidine move, leading to an unproductive configuration in which a water molecule links the catalytic triad residues via hydrogen bonds (Larsen *et al.*, 2001). In AEH such a bridging water molecule has not yet been found.

Three-dimensional structure of AEHs

The crystal structures of AEHs from *X. citri* and *A. turbidans* (Barends *et al.*, 2003b; Barends *et al.*, 2004) showed that the enzymes are tetramers composed of two dimers. Within one dimer, two subunits embrace each other with their N-termini (the first 20 residues after the signal sequence), which apparently are responsible for dimer formation. Although one subunit contains a complete active site that is functional in the dimer (*Chapter 4*), the separate subunits are not active (Kato and Kakinuma, 1980) and thus multimerization is needed for activity. Since in the dimer the active sites do not seem to interact with each other or with the dimer interface, dimerization must influence the catalysis indirectly, for example by stabilizing the oxyanion hole as is observed for the human cytomegalovirus protease (Batra *et al.*, 2001). Structural and kinetic analysis of mutants with mutations at the dimer interface of the N-terminal arms might reveal the molecular mechanism that underlies the activation upon dimerization.

Each monomer consists of an N-terminal α/β-hydrolase fold with a cap domain followed by a β-strand-rich jellyroll domain (Fig. 2 and *Chapter 5*). The predicted secondary structure elements for AEH from *A. turbidans* (*Chapter 3*) appeared in good agreement with its 3D structure, especially within the α/β-hydrolase fold (Fig. 2). In 1992, the α/β-hydrolase fold was identified as a specific fold of helices and sheets (Nardini and Dijkstra, 1999; Ollis *et al.*, 1992), and nowadays, it is the largest known superfamily of related enzymes with 22 separate families from which the structures have been solved (SCOP database (Murzin *et al.*, 1995)). The elucidation of the structure of the AEHs added another member to this family.

The α/β-hydrolase fold displays an immense flexibility as it tolerates variable insertions between the strand and helices, so that the domain size can vary from 181 residues in the lipase from *Bacillus subtilis* (Pouderoyen *et al.*, 2001) to 582 residues in mouse acetylcholinesterase (Heikinheimo *et al.*, 1999). Sometimes the insertions at the C-terminal ends of strands are big enough to form an additional domain. A very well known additional domain is the so-called cap domain, which is usually located between strand 6 and strand 7 and is also found in the AEHs. The cap domain is often involved in the activation of the enzyme, opening or closing of the active site of an α/β-hydrolase fold enzyme, for example in lipases (Miled *et al.*, 2000). The cap domain can also be important for interaction with the substrate. For example, in the crystal structure of cocaine esterase complexed with the product, the cap domain has 18 van der Waals contacts with the substrate (Larsen *et al.*, 2001). Additionally, in haloalkane dehalogenase mutations in the cap domain led to weakened interaction with the halogen atom or halide ion in the active site (Krooshof *et al.*, 1998). In the crystal structure of AEH we also see interactions of residues from the cap domain with the substrate

(Met200, Trp209 and Asp219, *Xanthomonas* numbering, *Chapter* 5). Sequence alignments of the AEHs from *X. citri* and *Z. mobilis* with the AEH from *A. turbidans* revealed that a residue in the cap domain is possibly involved in the ability to accept a *para*-hydroxy substituted substrate. In the crystal structure of *A. turbidans* AEH Ala250 is found in close proximity of the site where a *para*-hydroxy group is expected. In *X. citri* a more bulky Asn (Asn219) replaces this alanine (Barends *et al*., 2004). However, an Ala is also present in *Z. mobilis*, which is not able to convert *para*-hydroxyl substituted β-lactam substrates either. Sequence alignments reveals several positions differing only in *A. turbidans*. In combination with the structure two were found in the neighborhood of the acyl-binding pocket. In particular, the replacement of a hydrophobic Trp in *X. citri* (Trp267) and *Z. mobilis* (Trp249) by a smaller and more hydrophilic Phe in *A. turbidans* (Phe298) was found. At the same time a Leu (Leu671 in *X. citri* and Leu289 in *Z. mobilis*) is replaced by a Met in *A. turbidans* (Met302). The combination of these mutations might change the acyl binding pocket in such a way that a more bulky hydroxyl substituted side chain is accepted by *A. turbidans*.

The α/β-hydrolase fold domain frequently interacts with other domains. For example in prolyl oligopeptidase, the α/β-hydrolase fold of 8 strands is preceded by 354 amino acids that form a 7-bladed β-propeller structure that is positioned above the active site and selects substrates by size exclusion (Fülöp *et al*., 1998). In AEH the α/β-hydrolase fold works side by side with a β-jellyroll domain. Presently, the function of this domain is unknown, although an important role in maintaining the overall tertiary structure has been suggested for the one in cocaine esterase (Larsen *et al*., 2001). In AEH it influences the substrate specificity via residue Trp465, which stabilizes the cluster of negative charges (the α-amino binding pocket) through a hydrogen bond of the indole nitrogen with the side chain oxygen of Glu309. Structurally, the jellyroll domain is assigned to the galactose-binding domain-like superfamily (SCOP (Murzin *et al*., 1995)). In this superfamily the jelly roll domain is found N- or C-terminally, and is often observed within carbohydrate binding modules. For the AEH from *P. melanogenum* (reclassified as *X. maltophilia*) it has been reported that it possessed 13% (w/w) carbohydrate. This might indicate that the AEHs are glycosylated or that the jellyroll domain is involved in carbohydrate binding, although for now it is unclear to what purpose.

A new class of β-lactam acylases

The β-lactam acylases are traditionally classified according to their preferred antibiotic in hydrolysis reactions. However, structural analysis of PA already showed that the interaction with the β-lactam nucleus is much less than with the acyl group. The same was observed in the structure of *X. citri* AEH where modeling of ampicillin in the active site revealed more (possible) interactions with the side chain than with the β-lactam nucleus (*Chapter 5*). The higher level of interactions with the acyl group is consistent with the alternative classification of the β-lactam acylases (proposed in 1985), which is based on the side chain preference as the main determinant (see Introduction). This would define the AEHs as class III of the α-acylamino-β-lactam acyl-hydrolases, of which the AEHs are the first to be characterized. However, with the sequence and

structural information available nowadays, a classification based on substrate specificity is obsolete since a classification based on sequence and structure gives more information about the evolutionary and mechanistic relationship of enzymes. From that point of view the AEHs disclose a new class of β-lactam acylases as they belong to a completely different structural family (α/β hydrolase fold) than the other known β-lactam acylases (Ntn-hydrolases). The glutaryl 7-ACA acylase from *B. laterosporus* also belongs to this α/β-hydrolase fold class of β-lactam acylases, as apparent from the predicted organization of the structural elements and the presence of the conserved catalytic residues (*Chapter 4*).

Biological function of AEHs

There is not much known about the biological function of the β-lactam acylases. The periplasmic penicillin acylase from *E. coli* is believed to be involved in the assimilation of phenylacetylated compounds as alternative carbon sources when the organism is outside its usual intestinal habitat. This function was derived from its induced expression in the presence of phenylacetic acid and the localization of the PA gene in close proximity of a 4-hydroxy-phenylacetic acid degradative pathway. It has been postulated that the PA gene is an evolutionarily recent acquisition of the *E. coli* strain to increase its catabolic versatility (Prieto *et al*., 1996).

The AEHs are very likely constitutive proteins as their expression is not induced by the presence of phenylacetic acid or phenylglycine methyl ester and activity was found in the wild-type organisms both when cultivated on rich and on minimal medium (unpubilished results). The large inserts in the cosmids of the AEH positive clones from the genome libraries of *X. citri* and *A. turbidans* made it possible to sequence the ORFs up- and downstream of the *aeh* genes, which might give clues about the biological function of the AEHs. All the ORFs found upstream of the AEH genes of *A. turbidans* (*Chapter 3*), *X. citri* (*Chapter 4*) and *Z. mobilis* (a succinylarginine dihydrolase involved in arginine metabolism) encode proteins that are involved in the biosynthesis of amino acids. Although no direct relation between the encoded enzymes and the AEHs could be found, it may suggest a supporting role in general amino acid biosynthesis for the AEHs. Alternatively, from the structure we learned that the active sites of the individual monomers are located in a central cavity that is only accessible through narrow holes in the spherical tetramer. Obviously, these holes restrict the substrate size, which possibly protects folded polypeptides against the amidase activity of AEH. Thus, the tetrameric structure of the AEHs suggests that they may serve to hydrolyse misfolded oligopeptides. In the genome sequencing projects of the *Xanthomonas* strains the AEHs have been categorized in the cluster of proteins involved in pathogenicity, virulence and adaptation, more specifically in toxin production and detoxification. In conclusion, there is no consensus about the cellular role of the AEHs.

FUTURE RESEARCH

The efficiency of a kinetically controlled synthesis reaction for semi-synthetic β-lactam antibiotic is determined by the kinetic properties of the enzyme. Important parameters are the rate of conversion of the acyl donor vs. that of the product (factor α, *Chapter* 6) and the relative rate of acyl transfer to water (hydrolysis) and to a β-lactam nucleophile (aminolysis).

The relative rate of aminolysis can be favored through lowering the water activity by performing the reaction in mixed or pure organic solvents or by adding water-activity-depressing agents. In the presence of methanol (10-40%) (Fernandez-Lafuente *et al*., 2001; Nam *et al*., 2001), glycerol (15%), sucrose (30%), sorbitol (20%) (Hyun *et al*., 1993b) or polyethylene glycol (25%) (Hyun *et al*., 1993a) up to a 2.5-fold increase in the conversion yield was reached for the AEH of *X. citri*. However, enzymatic activity in the presence of 16% methanol or more requires some form of stabilization of the protein (Fernandez-Lafuente *et al*., 2001; Nam *et al*., 2001). With the sequence and structure in hand, one could locate possible flexible regions within the AEHs and incorporate disulfide bridges at these positions. Other ways to stabilize enzymes are to form insoluble cross-linked enzyme aggregates (CLEAs), enzyme crystals (CLECs) or immobilization on an inert carrier. AEHs have been stabilized by adsorption onto inert adsorbents like silica (Choi *et al*., 1981) and by chemical cross-linking with glutaraldeyde (Fernandez-Lafuente *et al*., 2001). The latter method increased the synthetic yield in the synthesis of ampicillin to 85%, and the stereospecificity for the acyl-donor phenylglycine methyl ester was improved as well, allowing the use of a racemic mixture of the activated side chain.

An alternative approach to increase the degree of aminolysis during the catalytic cycle is to mutate the enzyme. An example of this approach is the construction of thiosubtilisin, a mutant of the serine protease subtilisin in which the catalytic serine is mutated to a cysteine. With this mutant the preference for aminolysis over hydrolysis increased over a thousand fold (Abrahmsén *et al*., 1991). In view of these results we mutated the catalytic serine of the AEH from *A. turbidans* to cysteine by site-directed mutagenesis. Unfortunately, no detectable activity was measured with this mutant. This might indicate that the Ser205Cys mutant has lost its ability to transfer the acyl group to an acceptor as found for the analogous mutant of the myristoyl-ACP thioesterase (Li *et al*., 1996). Pre-steady state kinetic experiments, for example with *p*-NPGB, in which the acylation of the enzyme can be monitored, might confirm this. Another opportunity to increase the degree of aminolysis by site directed mutagenesis is to alter the binding site for water. In the structure of the AEHs, a binding site for the incoming nucleophile was identified next to the catalytic histidine (Ser371, *Acetobacter* numbering). Additionally, the β-lactam acyl acceptor is expected to interact with TyrB (Tyr112, *Acetobacter* numbering). The partly physical separation of the binding sites for water and that for the acyl acceptor or leaving group allows the optimization of the synthesis/hydrolysis ratio in the synthesis of β-lactam antibiotics via site-directed mutagenesis.

To obtain a more beneficial affinity of the enzyme for the acyl donor than for the product the binding with the β-lactam nucleus needs to be lowered. An obvious way to accomplish this is to alter the acyl acceptor and leaving group binding site, Tyr112. In the inactive Ser205Ala mutant of *A. turbidans* AEH co-crystallized with ampicillin, Tyr112 has interactions with the β-lactam moiety of ampicillin. Other residues having interactions with the β-lactam nucleus are Tyr375, Ser371, Arg117 and Glu207 (Barends *et al*., 2004). These residues are good candidates for site-directed mutagenesis to obtain better synthesizing enzymes, although the effects might be more

complex than expected from their roles described above. Mutation of Tyr206, located next to the nucleophile and involved in stabilizing the oxyanion formed during the transfer of the acyl group via its backbone NH (*Chapter 4* and *5*), showed that improvement is possible. The specificity (k_{cat}/K_M) of the Tyr206Asn mutant towards the acyl donor increased while the specificity toward the product (antibiotic) decreased. This resulted in an overall improved cephalexin synthesis reaction, including i) a higher maximal product accumulation (1.6-fold increase in Q_{max}), ii) a higher ratio between the initial rate of aminolysis and of hydrolysis (1.5-fold increase), and iii) an increased ratio between the formation of synthesis product over hydrolysis product at Q_{max} (1.5-fold increase). The experiments and recently solved crystal structures of *A. turbidans* wild-type AEH, Tyr206Ala and Ser205Ala mutant AEH showed that the tyrosine has a mutiple role. Besides the stabilization of the oxyanion, it indirectly influences substrate binding, since mutations at this position influenced the K_M of both the antibiotic and acyl donor (*Chapter 6*). Starting with the Tyr206Ala and/or Tyr206Asn mutant(s) in directed evolution experiments may lead to further improved enzymes.

An obvious way to change the substrate specificity of the AEHs is to mutate the residues forming the carboxylate cluster. Maybe the α-amino binding pocket formed by the aspartates can be altered in such a way that a Cα-hydroxyl group is more readily accepted (cefamandole) or that a more bulky group is accepted at that position (like in N-carbamoyl-D-*p*-hydroxy-phenylglycine, making a decarbamoylation to produce D-*p*-hydroxyphenylglycine superflous). The glutamate from the carboxylate cluster aligns with Phe261 of cocaine esterase that interacts with the bound inhibitor in the crystal structure of cocaine esterase (Larsen *et al.*, 2001). Despite the different substrate ranges of these two enzymes there seems to be conservation of the residues involved in substrate binding. Therefore other residues aligning with residues that interact with the inhibitor in cocaine esterase might be interesting to mutate in AEH as well, like Trp166, Leu 407 and Phe408 from cocaine esterase that correspond to Thr224, Asp458 and Phe456 in *X. citri* AEH.

An emerging challenge is to accomplish full biosynthesis of semi-synthetic β-lactam antibiotics in so-called microbial cell factories (Bruggink, 2001). A proof of principle for this approach has been given with the integration of an expandase gene from *Streptomyces clavuligerus* into a *Penicillium* strain, which leads to production of cephem nuclei (Robin *et al.*, 2001; Velasco *et al.*, 2000). Maybe in the future the AEHs could be integrated in a system to accomplish coupling of α-amino side chains to different nuclei.

In conclusion, there are still a lot of opportunities for challenging fundamental and applied research on the biotechnological interesting AEHs. Hopefully, in the future the knowledge about the structure-function relationship of the AEHs can be increased facilitating the development of a tailor made biocatalyst.

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INLEIDING VOOR ALGEMEEN PUBLIEK

Een aantal basisbegrippen

Katalyse

Een chemische reactie tussen verschillende stoffen, zoals die kan plaatsvinden op de labtafel, in een fabriek, of in een levend organisme, verloopt meestal niet spontaan. De stoffen moeten over een barrière heen worden geholpen, ze moeten geactiveerd worden, wat bijvoorbeeld kan door het reactiemengsel te verhitten. Een andere manier om een reactie om gang te brengen is het toevoegen van een zogenoemde katalysator. Een katalysator is een stof die de energiebarrière verlaagt zodat een chemische reactie sneller kan verlopen.

Katalysatoren (o.a. platina) worden bijvoorbeeld in katalysatoren van auto's gebruikt om schadelijke gassen die vrijkomen bij de verbranding van benzine, zoals koolstofmonoxide (giftig) en stikstofdioxide (veroorzaker zure regen en smog), om te zetten in onschadelijke gassen zoals koolstofdioxide en stikstof (koolstofdioxide draagt helaas wel bij aan het broeikaseffect en dus blijft autorijden ondanks de katalysator milieu-onvriendelijk, zij het in mindere mate).

Een *bio*katalysator is een katalysator van biologische oorsprong in de vorm van bijvoorbeeld een plant, een schimmel, een bacterie, of een eiwit. Eiwitten met een katalytische functie noemen we enzymen.

Enzymen

Enzymen zijn heel belangrijk voor al het leven op aarde aangezien ze verantwoordelijk zijn voor het grootste deel van de stofwisselingsprocessen die plaatsvinden in levende organismen, van bacteriën tot zoogdieren Zo zitten enzymen bijvoorbeeld in ons traanvocht om te helpen bacteriën af te breken en bevinden ze zich in onze darmflora om ervoor te zorgen dat zetmeel en eiwitten in ons voedsel verteerd worden. De naam van een enzym is vaak te herkennen omdat deze meestal eindigt op –ase, bijvoorbeeld amylase (breekt zetmeel af).

Enzymen zijn opgebouwd uit een lange keten van 20 verschillende aminozuren. De volgorde van de aminozuren en lengte van de keten, die vastgelegd zijn in het erfelijk materiaal (DNA) van een organisme, zijn voor elk soort enzym verschillend en bepalen de vorm en de functie van het enzym.

De kracht van enzymen schuilt in het feit dat ze bepaalde stoffen (substraten) optimaal ten opzichte van elkaar positioneren voor het maken of breken van chemische bindingen. De eerdergenoemde energiebarrière van reacties wordt dan sterk verlaagt, waardoor reacties soms wel een miljard keer sneller kunnen verlopen. Om dit te bewerkstelligen hebben enzymen een zogenaamde actieve holte waarin de substraten passen als een sleutel in een slot. Eenmaal juist gepositioneerd gaat het enzym een interactie aan met de substraten (wat meestal gebeurt door een samenspel van een aantal verschillende aminozuren). De krachten die zo ontstaan helpen de substraten met elkaar te reageren. Na de reactie verlaten de producten de actieve holte en keert het enzym terug naar zijn oorspronkelijke vorm zodat het klaar is voor een nieuwe ronde van katalyse.

Mensen hebben onbewust al heel lang gebruik gemaakt van enzymen, bijvoorbeeld bij het maken van brood of het brouwen van bier. De gistcellen die bij deze processen worden gebruikt bevatten enzymen die suikers omzetten. Echter, pas in het begin van de 20ste eeuw ontstond enig inzicht in de aard en werking van enzymen en

ontwikkelde men methoden om ze te isoleren zodat ze ook buiten het organisme ingezet konden worden. Sindsdien zijn er tal van toepassingen voor enzymen bijgekomen; we vinden ze nu ook in wasmiddelen waar ze o.a. helpen bij het afbreken van vet (lipases), of in tandpasta waar ze het speeksel helpen gaatjes te voorkomen (o.a. glucoseoxydases) of worden ze gebruikt bij het bleken van jeans (cellulases).

Door de grote katalytische kracht van enzymen zijn ze ook zeer interessant voor de chemische industrie. Bijkomende voordelen van het inzetten van enzymen zijn onder anderen dat ze a) zeer specifiek zijn in het uitvoeren van de gewenste reactie, b) geen bijproducten geven waardoor een proces efficiënter kan worden uitgevoerd, c) geschikt zijn voor hergebruik en d) hun reactie uitvoeren in een waterig milieu (geen organische oplosmiddelen nodig). Sinds de eerste toepassingen van enzymen in een chemisch proces in de jaren 80 zijn veel chemische bewerkingen omgezet naar 'groene' milieuvriendelijke enzymatische varianten, hetgeen heeft geleid tot een aantal zeer efficiënte, kosten- en afval-besparende processen. De mogelijkheid tot het toepassen van enzymen (biokatalyse) heeft dan ook de constante interesse van de industrie, en inmiddels gebruikt men enzymen bij de bereiding van suikers en andere zoetstoffen, hormonen, antibiotica en diverse geneesmiddelen.

Dit proefschrift beschrijft de karakterisatie van enzymen, de α-aminozuur-ester hydrolases, die gebruikt kunnen worden bij het maken van semi-synthetische β-lactam antibiotica. Een inleiding tot deze veel gebruikte antibiotica en een beschrijving van de bereikte resultaten kunt u lezen in de Nederlandse samenvatting.

NEDERLANDSE SAMENVATTING

β-lactam antibiotica

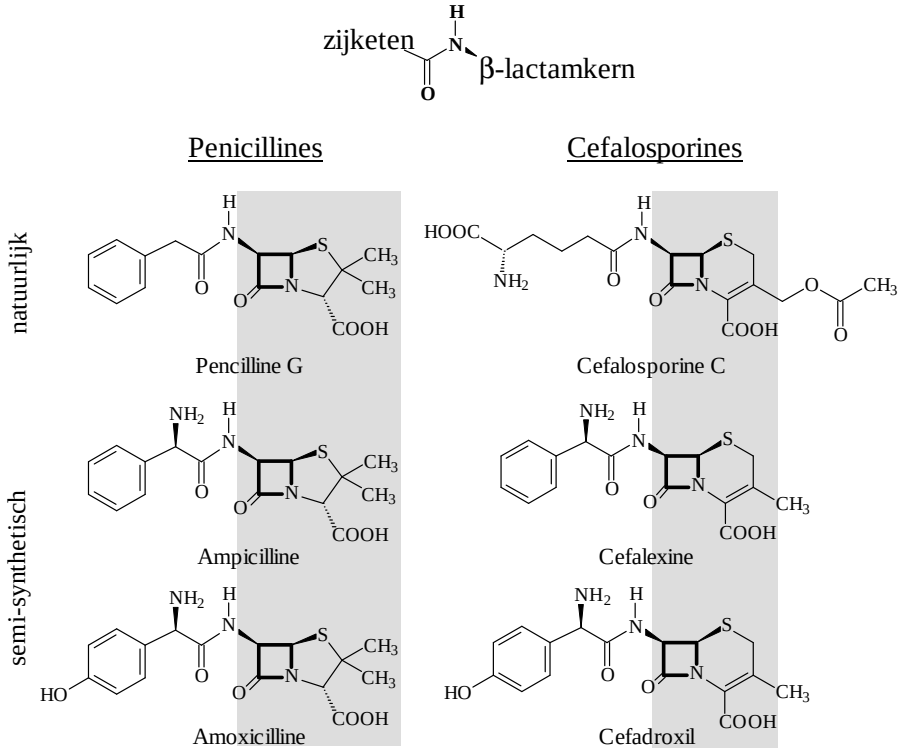
Eén van de meest voorgeschreven soort antibiotica zijn de β-lactam verbindingen, waarvan penicilline-G wel de bekendste is (Fig 1). Sinds het op grote schaal beschikbaar komen van deze groep antibiotica in de jaren 40 zijn daarmee vele mensenlevens gered van bacteriële infecties. Helaas zijn door intensief en onzorgvuldig gebruik in de loop der jaren verscheidene bacteriën resistent geworden voor penicillines en ontstond de behoefte aan antibiotica met andere eigenschappen. De bepaling van de moleculaire structuur en de daarmee gepaard gaande ontdekking van de biologisch actieve β-lactam ring hebben het mogelijk gemaakt antibiotica met nieuwe eigenschappen te produceren (Fig. 1). Men ontdekte dat de effectiviteit van het antibioticum voornamelijk wordt bepaald door de zijketen die aan de bicyclische β-lactam groep gekoppeld is (Fig. 1). Door de natuurlijke zijketen te vervangen coor een synthetische variant ontstaan antibiotica met een ander bacterieel werkingsspectrum en andere farmacologische eigenschappen. Voor de productie van dergelijke semi-synthetische antibiotica wordt allereerst penicilline G gesplitst in de β-lactam-kern en de zijketen. De verkregen kern wordt vervolgens gekoppeld worden aan een geschikte synthetische zijketen (Fig. 1). De in 1948 ontdekte cefalosporine-kern, waarin de β-lactam ring is gefuseerd met een dihydrothiazine ring in plaats van een thiazolidine ring (Fig. 1), vergrootte de mogelijkheden voor het maken van nieuwe β-lactam antibiotica nog verder.

Biokatalyse in de productie van semi-synthetische β-lactam antibiotica

Het overgrote deel (2/3) van de jaarlijks geproduceerde penicilline G (33.000 ton) wordt gesplitst in kern en zijketen om vervolgens de kern te gebruiken voor de bereiding van semi-synthetische β-lactam antibiotica. Het chemisch verbreken van de amidebinding tussen kern en zijketen in penicilline-G is een efficiënte maar een milieubelastende en gecompliceerde procedure, mede door de zeer reactieve maar onmisbare β-lactam ring. De ontdekking en het op grote schaal beschikbaar komen van enzymen (β-lactam acylases) die specifiek de koppelende amide-binding (Fig. 1) hydrolyseren heeft uitkomst geboden.

De β-lactam acylases zijn aan de hand van hun voorkeur voor een bepaald antibiotica in de hydrolyse ingedeeld in vijf klassen (zie *Hoofdstuk 1*). Vergaande karakterisatie heeft aangetoond dat al deze enzymen (behalve de ampicilline acylases, waarvan tot recentelijk geen structuur of aminozuurvolgorde bekend was) behoren tot dezelfde familie van enzymen, de zogenoemde N-terminale-nucleofiel (Ntn) hydrolase superfamilie. Inmiddels is in de industrie de chemische hydrolyse van penicilline G, dat gemaakt wordt door fermentatie van de schimmel *Penicillium chrysogenum*, geheel vervangen door een biokatalytische variant, waarbij penicilline-G acylase van *Escherichia coli* (PA) wordt gebruikt.

PA kan ook gebruikt worden om geschikte nieuwe synthetische zijketens te koppelen aan vrije β-lactam kernen. Andere enzymen die ook in staat om deze koppeling uit te voeren zijn de α-aminozuur ester hydrolases (AEHs). Deze enzymen hebben een voorkeur voor α-amino



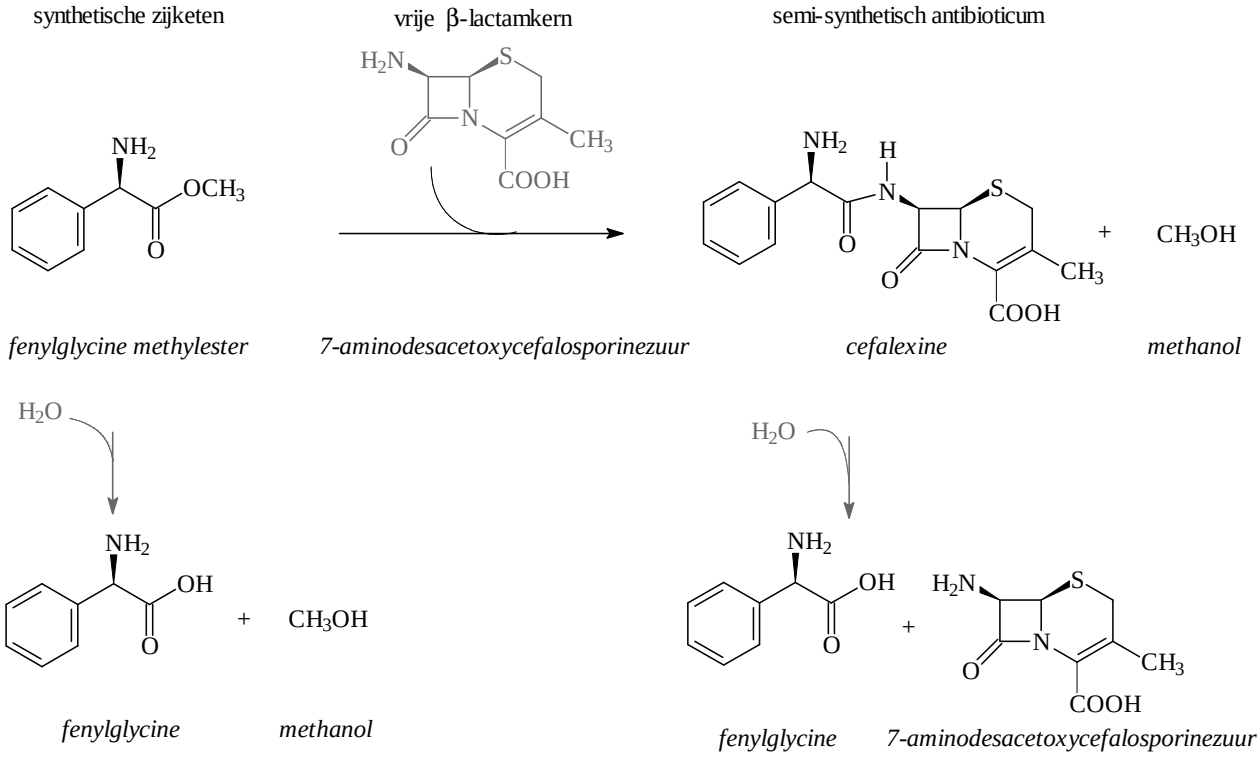
Figuur 1. Chemische structuur van een aantal β-lactam antibiotica. Penicilline G en cefalosporine C zijn natuurlijke β-lactam antibiotica, de overigen zijn semi-synthetische β-lactam antibiotica. Links een aantal penicillines met een 6-aminopenicillaanzuur β-lactamkern (in het grijze vlak links) en rechts een aantal cefalosporines met een cefalosporine β-lactamkern (grijze vlak rechts). De belangrijke β-lactam ring is vet gedrukt. De te splitsen amidebinding tussen zijketen en β-lactamkern is in de bovenste figuur uitgelicht. Het verschil tussen penicilline G and ampicilline is de aanwezigheid van de α-aminogroep. De α-aminozuur ester hydrolases hebben deze groep nodig voor activiteit

gesubstitueerde synthetische zijketens (zoals aanwezig in cefalexine en ampicilline (Fig. 2), ze werden in het verleden dan ook als ampicilline acylases geclassificeerd). Beide enzymen katalyseren de koppeling vanuit de (als amide of ester) geactiveerde synthetische zijketens en de vrije β-lactam kernen. Echter, ze katalyseren ookongewenste bijreacties: de hydrolyse van de geactiveerde zijketen en van het product (Fig. 2). Het is dan ook de uitdaging voor een biochemicus om uit te vinden hoe het enzym werkt en vervolgens aan te geven hoe de ongewenste hydrolyse-reacties te voorkomen dan wel te verminderen zijn in verhouding tot de gewenste koppelings-reactie.

Ondanks de mogelijke voordelen van de toepassing van de AEHs bij de koppelings-processen voor de synthese van α-amino gesubstitueerde β-lactam antibiotica (zie *Hoofdstuk 1*) heeft de betere beschikbaarheid en uitgebreidere kennis van PA er destijds (eind jaren 90) toe geleid dat PA is toegepast bij de eerste enzymatische productie van een semi-synthetisch antibioticum (cefalexine). Echter ook hier is altijd behoefte aan verbetering van het proces en dus is besloten om te onderzoeken of andere enzymen, zoals de AEHs, ook geschikt zouden kunnen zijn voor een dergelijk proces.

EEN SAMENVATTING VAN HET WERK BESCHREVEN IN DIT PROEFSCHRIFT

Om na te gaan welk enzym het beste is voor het maken van α-amino gesubstitueerde semi-synthetische antibiotica en vooral voor de synthese van ampicilline, hebben we een twaalftal β-lactam acylases (waaronder verschillende penicilline acylases en een vijftal AEHs) vergeleken wat betreft hun vermogen de synthese van ampicilline te katalyseren (*Hoofdstuk 2*). Drie AEHs kwamen er als beste uit, de AEHs van de micro-organismen i) *Acetobacter pasteurianus* ATCC 6033, ii) *Acetobacter turbidans* ATCC 9325 en van iii) *Xanthomonas citri* IFO 3835. Om verdere karakterisatie van deze enzymen mogelijk te maken is in de regel een grote hoeveelheid enzym nodig. Helaas is het gehalte van deze enzymen in hun natuurlijke gastheer erg laag (gemiddeld ongeveer 0.02% van het totale eiwit), hetgeen het zuiveren ervan zeer arbeidsintensief maakt. Om toch voldoende enzym te kunnen produceren is besloten de genen die voor de afzonderlijke AEHs coderen (de *aeh*-genen) te kloneren. Hiertoe zijn genbanken van de desbetreffende organismen in *Escherichia coli* gemaakt. De AEH-coderende genen konden niet in de genbanken worden gevonden d.m.v. het testen op AEH-activiteit (o.a. cephalexine synthese) in de gastheer. Daarom zijn de AEHs



Figuur 2. Reacties gekatalyseerd door α-aminozuur ester hydrolase. De bovenste reactie laat de synthese van cefalexine zien vanuit de tot ester of amide geactiveerde synthetische zijketen en de vrije β-lactamkern. De twee grijze reactiepijlen wijzend naar beneden geven de ongewenste hydrolyse van de geactiveerde zijketen (links) en het product (het semi-synthetische antibioticum, rechts) weer. Het enzym reageert eerst met de geactiveerde zijketen waarbij methanol en een acyl-enzym intermediair worden gevormd Dit intermediair kan vervolgens reageren met een β-lactamkern (synthese) of met water (hydrolyse). De acylgroep wordt dan overgedragen aan deze zogenoemde acylacceptoren. In deze figuur zijn de acylacceptoren grijs weergegeven

van *A. turbidans* en *X. citri* gezuiverd en is van een segment de aminozuurvolgorde bepaald zodat er een DNA-probe gemaakt kon worden waarmee de *aeh*-genen in de genbanken d.m.v. Southern hybridisatie konden worden gelokaliseerd (*Hoofdstuk 3* en *5*). Op deze wijze zijn de genen coderend voor de AEHs van *A. turbidans* en *X. citri* geïsoleerd en is hun DNA-volgorde bepaald. Helaas lukte het niet om de AEH van *A. pasteurianus* voldoende te zuiveren voor het bepalen van een aminozuursequentie. Echter, met de DNA-probe die correspondeert met het *aeh*-gen van *A. turbidans* kon ook het *aeh*-gen van *A. pasteurianus* gevonden worden, dat achteraf gezien vrijwel niet bleek te verschillen van het gen van *A. turbidans*.

α-Aminozuur ester hydrolases

De *aeh*-genen van *A. turbidans* en van *X. citri* coderen voor eiwitten met ketens van respectievelijk 667 en 637 aminozuren. Twee (dimeer) of vier (tetrameer) van deze aminozuurketens (subunits) vormen een actief enzym met twee dan wel vier actieve holtes (*Hoofdstuk 3, 4 en 5*). Uit vergelijkingen van de verkregen aminozuurvolgordes met volgordes in databases bleek dat de AEHs geen homologie vertonen met bekende β-lactam acylases zoals PA die tot de zogenoemde Ntn hydrolases behoren. Er werd wel 28% volgorde-identiteit gevonden met een glutaryl acylase van *Bacillus laterosporus*. Van dit enzym is geen structuur bekend en ook de aminozuurvolgorde van dit eiwit vertoont geen overeenkomsten met andere β-lactam acylases. Daarentegen bleek dat het eerste deel van de AEHs (ongeveer de eerste 40 tot 440 aminozuren) homologie vertoont met zogenoemde α/β-hydrolase-fold enzymen. De specifieke opvouwing in deze enzymen zorgt voor de juiste positionering van de voor katalyse belangrijke aminozuurresiduen, die een katalytische triade vormen. In de AEHs is de triade opgebouwd uit een serine, histidine en aspartaat (*Hoofdstuk 3 en 4*). Door deze aminozuurresiduen te veranderen in een niet katalytisch actief aminozuur (d.m.v. mutagenese) werd hun onmisbare rol bevestigd. Massaspectrometrie-experimenten lieten zien dat de acylgroep van een remmer die een geactiveerde synthetische zijketen nabootst, covalent gebonden wordt aan het enzym wanneer het enzym met de remmer reageert (*Hoofdstuk 4*). Dit geeft aan dat de enzymatische reacties verlopen via de vorming van een zogeheten acyl-enzym intermediair. Bij de synthese van antibiotica wordt de acylgroep via het enzym doorgegeven aan een in het reactiemengsel aanwezige acceptor, de β-lactam kern. Dit leidt tot de vorming van het gewenste semi-synthetische β-lactam antibioticum. De acylgroep kan echter door het acylenzym-intermediair ook overgedragen worden aan een watermolecuul, hetgeen leidt tot een ongewenste bijreactie, nl. de hydrolyse van de acyldonor (Fig. 2).

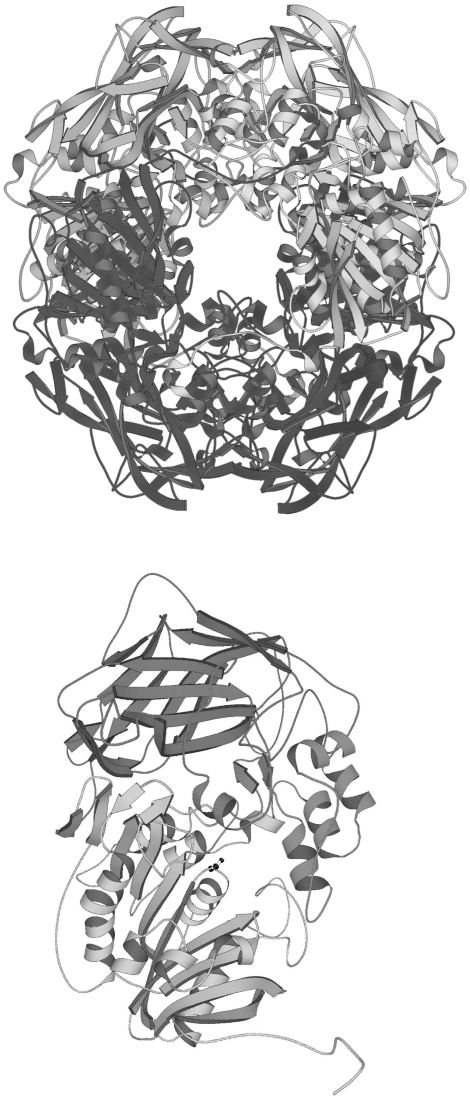
Met het bepalen van de driedimensionale structuur van de AEH van *X. citri* (Fig. 3) ontstond meer inzicht in de binding van de afzonderlijke substraten. De aanwezigheid van een substraatmolecuul (ampicilline) in de actieve holte onthulde een aantal mogelijke interacties tussen substraat en specifieke residuen van het enzym (*Hoofdstuk 5*). Zo bleek dat de doorgaans positief geladen α-aminogroep precies paste in een negatief geladen deel van de holte die gevormd wordt door de carboxylaatgroepen van de zure zijgroepen van een drietal aminozuren. Dit verklaart de hoge specificiteit van de AEHs voor substraten met een α- aminogroep. Deze en andere interacties kunnen in de toekomst belangrijk zijn

wanneer men de eigenschappen van het enzym wil veranderen.

In Hoofdstuk 6 hebben we alvast een voorproefje genomen op de mogelijkheid door mutaties de eigenschappen van de AEHs te veranderen dan wel te verbeteren. Daartoe hebben we zes maal een aminozuur dat indirect betrokken is bij de katalyse (oxyanion-holte) vervangen door een ander aminozuur. Mutatie van dit residu naar het aminozuur asparagine zorgde ervoor dat de affiniteit van het enzym voor het product verlaagd werd (*Hoofdstuk 4*). Dit is gunstig omdat het veranderde enzym minder geneigd is het product te hydrolyseren en er dus een hogere productopbrengst kan optreden. Deze mutant produceerde inderdaad 1.6 keer meer cefalexine in een koppelingsreactie dan het oorspronkelijke enzym.

Nieuwe klasse van β-lactam acylases

Het zoeken in databases naar eiwitten die homoloog waren met de AEH-aminozuurvolgordes leverden een 7-tal eiwitten op waarvan de aminozuurvolgorde voor 60% of meer gelijk was aan die van één van de gekloneerde AEHs (Fig. 4). Onderlinge vergelijkingen van de aminozuurvolgordes lieten zien dat de katalytische residuen en de residuen die de negatieve holte voor de α-amino groep vormen geconserveerd zijn, waardoor we verwachten dat al deze eiwitten AEH-activiteit bezitten. Om deze hypothese te toetsen is het gen dat het vermoedelijke AEH enzym van *Zymomonas mobilis* codeert gekloneerd en tot overexpressie gebracht (*Hoofdstuk 5*). Experimenten lieten zien dat dit enzym inderdaad in staat is β-lactam antibiotica te produceren en dat het evenals de AEHs een α-aminogroep op het substraat nodig heeft voor activiteit (*Hoofdstuk 5*). Vergelijking van de



Figuur 3. De driedimensionale structuur van de AEH van *X. citri*. A) De tetrameer met 4 afzonderlijke subunits (elk een andere tint grijs). **B)** Eén subunit met links onder het α/β-hydrolase fold domein, met rechts (iets donkerder grijs het 'cap'-domein en bovenin (donkergrijs) het 'jelly-roll'-domein. De katalytische serine is m.b.v. balletjes en stokjes weergegeven

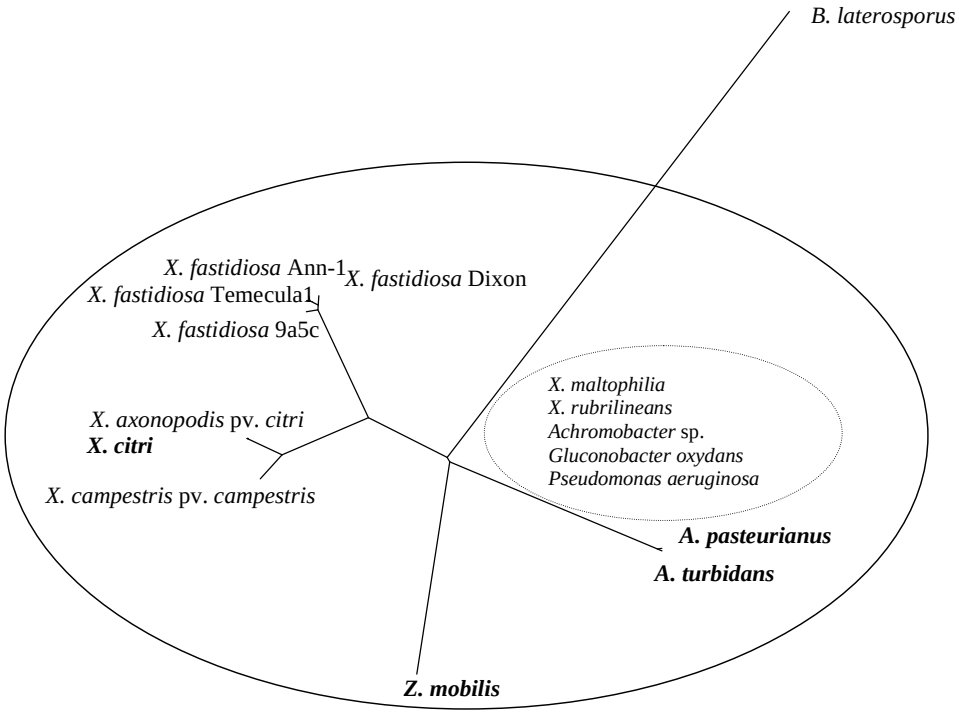
bekende AEH aminozuurvolgordes met die van verschillende eiwitten die qua volgorde op AEHs lijken, kan samen met structuuranalyses leiden tot de identificatie van aminozuren die verantwoordelijk zijn voor het bepalen van het substraatbereik. Door deze vervolgens te muteren en de mutant te karakteriseren kan meer inzicht

verkregen worden in de functionele rol van het desbetreffende residu. Er zijn in de literatuur nog een aantal AEHs genoemd, zoals die van *Pseudomonas melanogenum* (*Xanthomonas maltophilia*), *Achromobacter* sp., *Gluconobacter oxydans*, en een *Pseudomonas aeroginosa* (*Hoofdstuk 7*). Van deze eiwitten zijn geen aminozuurvolgordes bekend. Wel zijn ze opgebouwd uit subunits met ongeveer dezelfde lengte als de AEHs en vertonen ze dezelfde voorkeur voor substraten met een α-aminogroep. Experimenten hebben aangetoond dat van sommige AEHs de aminozuurvolgordes grote overeenkomsten vertonen met de bekende AEH van *X. citri* en dat ze dus naar alle waarschijnlijkheid tot de hier beschreven nieuwe klasse van β-lactam acylases behoren: de AEHs (Hoofdstuk *5*).

Conclusies

De α-aminozuur ester hydrolases zijn enzymen die in staat zijn semi-synthetische β-lactam antibiotica zoals ampicilline en cefalexine te maken.

Het werk in dit proefschrift heeft de beschikbaarheid van en de kennis over de AEHs aanzienlijk verbetert. Hun katalytische mechanisme en een aantal bijbehorende parameters zijn bepaald en de α-amino specificiteit is verklaard. Tevens is duidelijk geworden dat de AEHs α/β hydrolase fold enzymen zijn en daardoor een geheel nieuwe klasse van β-lactam acylases vormen. Verder is aangetoond dat deze enzymen interessante eigenschappen hebben voor biotechnologische toepassingen en dat er nog ruimte is om die aan de hand van specifieke doelen verder te optimaliseren.



Figuur 4. Weergave van de onderlinge relatie van de AEHs en gerelateerde eiwitten, een nieuwe groep β-lactam acylases. De eiwitten binnen de cirkel zijn voor 60% of meer identiek aan de AEHs. De AEHs van de vetgedrukte micro-organismen zijn in dit proefschrift gekloneerd en bestudeerd.

Zooooooooooooooooo, we zijn wel klaar, hè. Het heeft even geduurd, maar eindelijk ben ik toe gekomen aan het dankwoord (waarschijnlijk het meest gelezen stuk van het proefschrift). Allereerst wil ik Dick Janssen (mijn promotor) bedanken voor zijn vertrouwen en het uitspreken daarvan op de momenten dat ik het zwaar inzag. Jouw enthousiasme over dingen die we ontdekt hadden en het vervolgens aanreiken van nieuwe handvaten heeft mijn plezier in het onderzoek in die tijd zeker versterkt.

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